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Defining the role of Prox1 in Kaposi´s sarcoma herpesvirus life cycle

Krista Tuohinto

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University of Helsinki
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To my dad, for sparking my passion for science and for endless curiosity of things unknown, especially that of Cancer.

Abstract

Kaposi's sarcoma herpesvirus (KSHV), also called human herpesvirus 8 (HHV-8), was discovered following the AIDS-epidemic as the causative agent of Kaposi's sarcoma (KS), an angiogenic endothelial tumor of the skin, and of two rare lymphoproliferative diseases, primary effusion lymphoma (PEL) and multicentric Castleman disease (MCD). Infection by KSHV displays two life cycle phases; latent and lytic replication. In latency, the virus stays dormant within the host, expressing only a few genes and no viral particles are produced. Latency is the default mode of infection, however, upon appropriate induction the virus reactivates to express all of its genes and replicate viral DNA during the productive lytic replication, culminating with the release of infectious progeny particles and lysis of the host cells. Virus reactivation from latency to the lytic replication is an essential step in the KS pathogenesis.

Upon KSHV infection, endothelial cells (EC) undergo reprogramming towards spindle cell, the principal proliferating cell in advanced KS lesions. The transcription factor prospero related homeobox gene Prox1 has an important role in mediating the effects of KSHV on EC reprogramming, contributing to the KS development. Prox1 is the master regulator of lymphatic endothelial cell fate, and its expression is manipulated during the KSHV infection. However, the role of Prox1 in the KSHV life cycle and lytic reactivation has not been studied.

To elucidate the role of Prox1 in KSHV reactivation from latency, the effect of ectopic expression of Prox1 on the lytic gene and protein expression in both latent and reactivated KSHV-infected cells was studied. This led to a significant increase in KSHV lytic gene and protein expression, suggesting Prox1 as a positive regulator of KSHV lytic replication. Moreover, Prox1 wild-type, but not its DNA-binding deficient mutant, could significantly increase the release of infectious virions. To investigate the expression levels of Prox1 during KSHV infection, infection kinetics assay was performed, which showed an increase in the Prox1 levels during acute infection. Intriguingly, this was followed by a progressive decrease in the Prox1 levels as latency was established. In conclusion, the focus of this thesis is to investigate the role of Prox1 in KSHV reactivation, and to provide a deeper insight into the virus reactivation mechanisms that can be utilized for future therapeutic strategies against KSHV-mediated tumorigenesis of KS.

Keywords: KSHV, Kaposi's sarcoma, Prox1, virus reactivation, lytic replication

Abbreviations

AIDS	acquired immunodeficiency syndrome
BCBL1	body cavity-based lymphoma 1 (cell line)
BEC	blood endothelial cell
BSA	bovine serum albumin
Caki-1	clear-cell renal-cell carcinoma (cell line)
CD4	cluster of differentiation 4
Co-IP	co-immunoprecipitation
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
Dox	doxycycline
dsDNA	double stranded DNA
E	early
EBV	Epstein-Barr virus
EC	endothelial cell
EDTA	ethylenediaminetetraacetic acid
EndMT	endothelial-to-mesenchymal transition
FBS	fetal bovine serum
FLT4	fms related tyrosine kinase 4
GFP	green fluorescent protein
HAART	highly active antiretroviral therapy
HDAc	histone deacetylase
HEK293	human embryonic kidney 293 (cell line)
HHV-4	human herpes virus 4
HHV-8	human herpes virus 8
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HS	heparan sulfate

HUVEC	human umbilical vein endothelial cell line
IE	immediate early
IF	immunofluorescence
IgG	interleukin-6
IL-6	immunoglobulin G
kDa	kilodalton
KS	Kaposi’s sarcoma
KSHV	Kaposi’s sarcoma herpesvirus
L	late
LANA	latency-associated nuclear antigen
LEC	lymphatic endothelial cell
MCD	multicentric Castleman’s disease
miRNA	microRNA
MMP-9	matrix metalloproteinase-9
MMP-14	matrix metalloproteinase-14
mRNA	messenger RNA
MTA	mRNA transport and accumulation protein
MUT	mutant
NaB	sodium butyrate
O/N	overnight
ORF	open-reading frame
PAN	polyadenylated nuclear
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEL	primary effusion lymphoma
PFA	paraformaldehyde
Prox1	Prospero homeobox 1
pSIN	self-inactivating plasmid vector
Rb	retinoblastoma protein
RFP	red fluorescent protein
RNA	ribonucleic acid
RSV	Rous sarcoma virus

RT	room temperature
RTA	replication and transcription activator
SC	spindle cell
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
siRNA	short interfering RNA
STAT4	signal transducer and activator of transcription factor 4
TBS-T	Tris-buffered saline + Tween
TET	Tetracycline
U2OS	human osteosarcoma (cell line)
v-cyclin	viral cyclin
VC	vector control
VEGF	vascular endothelial growth factor
VEGF-R	vascular endothelial growth factor receptor
vFLIP	viral FLICE-inhibitory protein
vGPCR	viral G protein-coupled receptor
VSV-G	vesicular stomatitis virus coat G glycoprotein
WB	Western blotting
WT	wild-type

Contents

1 Introduction

To date, accumulating evidence suggests that tumor viruses contribute up to 15% of human cancers worldwide (Parkin 2006; de Martel et al. 2012). Even though infections by oncogenic viruses are common, the development of virus-induced malignancy is a rare consequence (Simpson et al. 1996). Complex host-virus interactions and other co-factors, both genetic and environmental, contribute to the emergence of cancers associated with viral infection (Ensoli and Sturzl 1998; Moore and Chang 2010). It is not evolutionarily advantageous for viruses to cause fatal cancer, as they are dependent on their host to survive and reproduce. Viral cancers can be considered as accidental manifestations resulting from loss of balance between the host and the virus, and just as deletorious to both.

Kaposi's sarcoma herpesvirus (KSHV) is recognized as one of the human tumor viruses, being the causative agent of Kaposi's sarcoma (KS) (Chang et al. 1994) and two rare lymphoproliferative disorders primary effusion lymphoma (PEL) (Cesarman et al. 1995) and multicentric Castleman's disease (MCD) (Soulier et al. 1995). KS is an angiogenic tumor of endothelial origin, widespread and fatal in patients with acquired immunodeficiency syndrome (AIDS) and geographically most prominent in some parts of Africa (Martin 2007; Mesri, Cesarman, and Boshoff 2010). However, the therapies against KSHV associated malignancies are limited and overall survival of the patients remains poor.

Hallmark of the oncogenic viruses is their prolonged persistence in infected individuals, allowing time for the multistep tumorigenesis to develop (Weinberg 1989; Moore and Chang 2010). Life-long infection of KSHV is achieved by establishment and maintenance of latency, a quiescent dormant state of the virus within the host cells (Ballestas, Chatis, and Kaye 1999). Sustained rate of reactivation causes the virus to initiate the replication of its own genome and the expression of all the viral genes. This lytic replication phase leads to production of new viral particles, and to death of the host cell is as virions are released (Renne, Zhong, et al. 1996; Miller et al. 1997; Staskus et al. 1997). The switch from latency to lytic replication is a crucial step in the pathogenesis of KSHV-induced cancer, and spontaneous reactivation contributes to progression of KS by

generation of virions to disseminate within the host. Furthermore, the lytic phase contributes to KS pathogenesis by inducing the production of cellular and virally-encoded inflammatory and angiogenic cytokines in the tumor microenvironment (Martin et al. 1999; Grundhoff and Ganem 2004). Therefore, the virus-host cell interplay has a crucial role in KS-induced oncogenesis, and a better understanding of the cellular factors able to affect KSHV lytic cycle and tumorigenesis is needed.

One of the host genes manipulated during KSHV infection is a transcription factor Prox1, the master regulator of lymphatic endothelial cell fate (Hong et al. 2004). KSHV infection of endothelial cells (ECs) triggers an aberrant differentiation process that leads to a deep reprogramming of those cells as well as to changes in their morphology, growth, angiogenic and invasive potential resulting in the characteristic spindle cells, which are the predominant proliferating cells in KS lesions and thus considered the KS tumor cells (Ganem 2010).

Substantial understanding of KSHV and its features in tumorigenesis has been acquired, even though the virus was discovered as the causative agent of KS only decades ago. The goal of developing more potential, rational targets for intervention of the KSHV associated diseases can be achieved by studying virus-host interactions and the life cycle of KSHV. Classic cancer therapies are generally used to treat KS patients, however, these therapeutic agents do not target the virus as latently-infected cells are insensitive to anti-herpesviral drugs which can target only viruses in the lytic phase, when the viral DNA is replicated (Kedes and Ganem 1997; Antman and Chang 2000). Since the fraction of viruses reactivating and entering the lytic cycle are susceptible to anti-viral therapy (Grundhoff and Ganem 2004), the identification of the cellular players that control herpesviral reactivation is of fundamental importance. Understanding the molecular mechanisms of KSHV reactivation is of interest to overcome the major obstacle of latency for therapies and perhaps in the future achieve eradication of this herpesvirus from its host. The role of Prox1 in virus-induced cell reprogramming has been studied (Hong et al. 2004; Wang, Trotter, et al. 2004; Yoo et al. 2012) but the role of Prox1 on lytic reactivation and replication is yet to be elucidated.

2 Review of the literature

2.1 Kaposi's sarcoma

2.1.1 Pathobiology of Kaposi's sarcoma

Kaposi's sarcoma was first described by a Hungarian dermatologist Moritz Kaposi in 1872 as "idiopathic multiple pigmented sarcoma of the skin" after discovering patch lesions on the skin of 5 of his patients (Sternbach and Varon 1995). At the time naming the neoplasm as sarcoma, indicating the similarities to traditional tumors of mesenchymal origin, has been reassessed due to unique characteristics of KS (Ganem 2010). In recent years, heterogeneity of cell types in various tumors has been noted in contrast to the idea of tumors as insular masses of proliferating cancer cells (Hanahan and Weinberg 2011). Histologically complex KS neoplasms display remarkable cell type diversity, depending on the stage of disease progression (Ganem 2006).

At the early stages KS appears as a flat patch lesion, without an evident tumor mass. In these early lesions, presence of inflammatory cells and dilated EC lined vascular spaces in irregular arrangements result in reddish coloration. Abundant neovascularization before development of solid tumors distinguishes KS from classical cancers that trigger inflammatory and angiogenic responses only at later stages of cancer progression. As the abnormal, leaky neovasculature expands through the dermis together with erythrocyte extravasation and haemosiderin accumulation, the lesions progress to plaque stage with induration and more intense red colour (Figure 1). Morphologically elongated cells, termed spindle cells (SC), arrange around the vascular spaces and aggregate forming large nodules. At advanced nodular stage, KS lesions consist mainly of SCs and of slit-like vascular spaces, lacking pericyte or smooth muscle coverage found in mature blood vessels. Consequently,

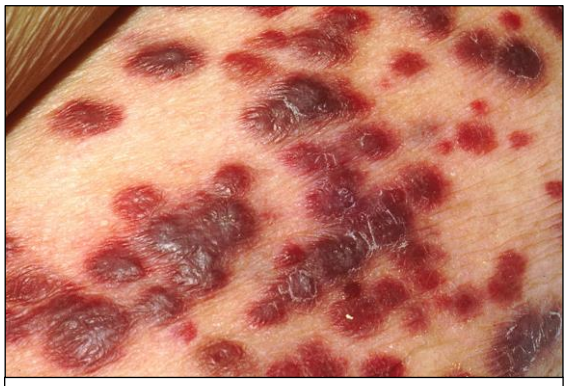

Figure 1. Kaposi's Sarcoma lesions on the skin of an AIDS-patient.
Courtesy of National Cancer Institute, 2001.

abnormal vessels are prone to leakage and rupture of blood causing haemorrhage and local edema resulting in dark red and violaceus KS nodules. (Gessain and Duprez 2005; Grayson and Pantanowitz 2008; Ganem 2010)

The heterogenic mass lesions are dominated by continuously proliferating neoplastic SC, accompanied by infiltrating inflammatory cells such as B and T cells, plasma cells, and monocytes, besides the extravasated erythrocytes (Ganem 2010). Contribution of inflammatory microenvironment is essential for KS tumorigenesis and promotes the establishment of the lesions. From the inception of KS lesion, proliferation of SC, angiogenic perfusion of vasculature, and inflammatory cytokines, all are necessary and participate to the disease progression (Ensoli and Sturzl 1998; Ganem 2010; Mesri, Cesarman, and Boshoff 2010). Distinct from traditional tumors, multifocal lesions of KS arise in the dermis in an independent manner (multicentricity) rather than through metastasis from primary tumor (Brooks 1986), and evidence supports that advanced KS lesions are polyclonal proliferations (Duprez et al. 2007). Consequently, KS evolves from red maculae (patches) through plaque-stage to nodules progressing locally or occur as widely diffused lesions in the patient (Gill et al. 1998; Ensoli et al. 2001). However, the clinical manifestation and morbidity of the disease is dependent on the particular epidemiological form of KS.

2.1.2 Epidemiological variants of Kaposi's sarcoma

Histopathological appearance of all the four recognized KS forms are similar, but however, distinguishable according to epidemiological characteristics (Grayson and Pantanowitz 2008). The first identified form is the classical KS primarily affecting elderly men of Mediterranean area and Eastern European Jewish descent. Slowly progressing and rarely life-threatening, this indolent tumor occurs locally most often on the skin of lower limbs (Brooks 1986). A more detrimental form is the endemic KS found in subequatorial Africa, and common amongst men and children. Similar to classic KS, endemic KS manifests primarily as localized plaques and nodules on lower limbs, but visceral involvement is more common than in the classical form and in children it is associated with a serious lymphoadenopathy (Slavin, Cameron, and Singh 1969; Taylor et al. 1972).

More aggressive forms of KS manifest in the immunodeficient patients as disfiguring lesions widespread on the skin of the body and face. The high morbidity and mortality of these KS cases results from the occurrence of the lesions in visceral organs, such as lungs and gastrointestinal tract, leading to serious and fatal complications causing respiratory failure, hemorrhage, diarrhea or obstruction (Friedman-Kien 1981; Dezube 1996). Iatrogenic KS among immunosuppressed transplant recipients was identified after recording the high incidence of KS in renal-transplant recipients and other patients receiving post-transplantation immunosuppressive therapy (Shepherd et al. 1997; Lebbe, Legendre, and Frances 2008). The fourth and the most notorious form recognized is the AIDS-associated KS, affecting immunosuppressed individuals with HIV infection advanced to AIDS.

KS did not attract much scientific or public attention until the 1980s when in the western countries the outbreak of AIDS caused dramatic increase in KS cases. KS became the most common HIV-associated fatal neoplasm, causing an extensive health burden (Friedman-Kien 1981; Haverkos and Drotman 1985). Implementation of highly active antiretroviral therapy (HAART) in 1996 improved the prognosis of patients with AIDS-associated KS considerably (Boshoff and Weiss 2002; Mocroft et al. 2004), and the incidence of AIDS-associated KS cases has rapidly declined in the western countries (Eltom et al. 2002; Ambinder and Cesarman 2007; Shiels et al. 2011). However, KS remains a significant health burden in sub-Saharan Africa due to persisting HIV epidemic and unavailability of antiretroviral therapy (Wabinga et al. 1993; Chokunonga et al. 2000).

As the malignancy was frequently manifesting in AIDS-patients, further studies on the role of HIV infection in KS pathogenesis were conducted. The association of KS and HIV infection was immense, and co-occurrence suggested HIV as a proximate cause. However, not all KS patients had HIV infection thus the notion of HIV as a main cause of KS was excluded (Beral et al. 1990). Classical and endemic forms of KS had no link to HIV infection, as no HIV proviral DNA was detected in the tumor biopsies. In addition, HIV-positive individuals had a varying risk of developing KS depending on the source of infection. KS occurrence was higher among homosexual or bisexual men with AIDS, compared to patients acquiring HIV through parenteral transmission, suggesting a link to

sexual transmission (Martin et al. 1998). Consequently, researchers prospected a yet unidentified sexually transmitted human pathogen as a causative agent of KS (Beral et al. 1990).

2.2 Kaposi's sarcoma herpesvirus

2.2.1 Discovery of the causative agent of KS

Chang and Moore, et al. discovered a novel herpesvirus from AIDS-KS biopsies the through representational difference analysis, isolating unique DNA sequences with no homologous sequence identified at the time in the current databases (Chang et al. 1994). The isolated DNA fragments of an unknown pathogen had similarities with the genomes of seven already known human herpesviruses (Longnecker and Neipel 2007). Consequently, the newly discovered virus was named human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma herpesvirus (KSHV) due to evidence linking it in the pathogenesis of KS. In several subsequent seroepidemiological studies, strong concordance between KSHV infection and the incidence of KS has been confirmed (Kedes et al. 1996; Miller et al. 1996; Gao et al. 1996). KSHV DNA is invariably present in the biopsies from all the forms of KS, and likewise emergence of KS is not observed in the absence of KSHV infection (Chang et al. 1994; Boshoff, Whitby, et al. 1995; Dupin et al. 1995). These results indicate the necessary role of KSHV infection in the KS development.

KSHV in the family of Herpesviridae is classified belonging to the subfamily of γ-2- herpesvirinae and is the first in the genus of *Rhadinoviruses* to infect humans (Russo et al. 1996; Moore et al. 1996). Close relation to a γ-1- herpesvirus Epstein-Barr virus (EBV) can be seen from the similar structure and genomic organization. EBV, also known as human herpesvirus 4 (HHV-4), is the aetiological agent of infectious mononucleosis, and the causal link between EBV and certain lymphomas have been revealed (Epstein, Achong, and Barr 1964; Henle, Henle, and Diehl 1968; Evans and Gutensohn 1984). Similar to KSHV, also EBV is recognized as an oncogenic human tumor virus.

2.2.2 KSHV seroprevalence mirrors the KS incidence

KSHV seroprevalence in the general population is less than 10% in Northern Europe, Asia and United States. In the Mediterranean region, where classic KS occurs, it varies between 10-30% and in the sub-Saharan Africa region, where endemic KS is highly diffused, the rate is more than 50% (Mesri, Cesarman, and Boshoff 2010). Regardless of the region, homosexual men have consistently been shown to have the highest seroprevalence (Martin et al. 1998). In concordance with the seroprevalence rates, incidence of KS is relatively rare in low prevalence areas in the general population compared to some regions of African countries (Figure 2) or among homosexual men.

Even though the seroprevalence rates are relatively common, the chance of developing KS for an individual in the general population is very low (Geddes et al. 1994; Hjalgrim et al. 1996; Iscovich et al. 2000). KSHV causes KS only in a minor fraction of infected hosts, indicating existence of additional co-factors. The KS risk is modulated by geographic, ethnic, genetic, immunologic and especially infectious cofactors. HIV infection increases the risk of developing KS considerably, as around 50% of KSHV and HIV double positive individuals will develop KS in 10-year period without HAART (Martin et al. 1998). Contribution of immunosuppression due to AIDS is immense, and represents the greatest co-factor in the KS pathogenesis (Boshoff and Weiss 2002). Among HIV-infected persons, the magnitude of immunosuppression, as evidenced by the declining CD4+ T-cell count, is predictive of development of KS (Dezube 1996; Krown, Testa, and Huang 1997; Renwick et al. 1998). Immunity impairment decreases the ability to maintain the cytokine homeostasis, and to respond to KSHV infection or KS tumor antigens (O'Connor and T-Scadden 2004). KSHV DNA increases in KSHV-positive patients as CD4+ count drops (McCune 2001; Tedeschi et al. 2001). Classical KS, having no link to HIV infection, has other cofactors involved in the disease progression. Genetic studies have reported increased risk in persons with higher interleukin-6 (IL-6) production or with differences in the human leukocyte antigen (HLA) complex participating in the regulation of immune system (Contu et al. 1984; Foster et al. 2000). Interestingly, a genetic predisposition to classical KS in Finland was reported in a genome-wide mutation analysis, describing rare heterozygous substitution in the DNA-

binding domain of signal transducer and activator of transcription factor 4 (STAT4) (Aavikko et al. 2015; Kaasinen et al. 2013).

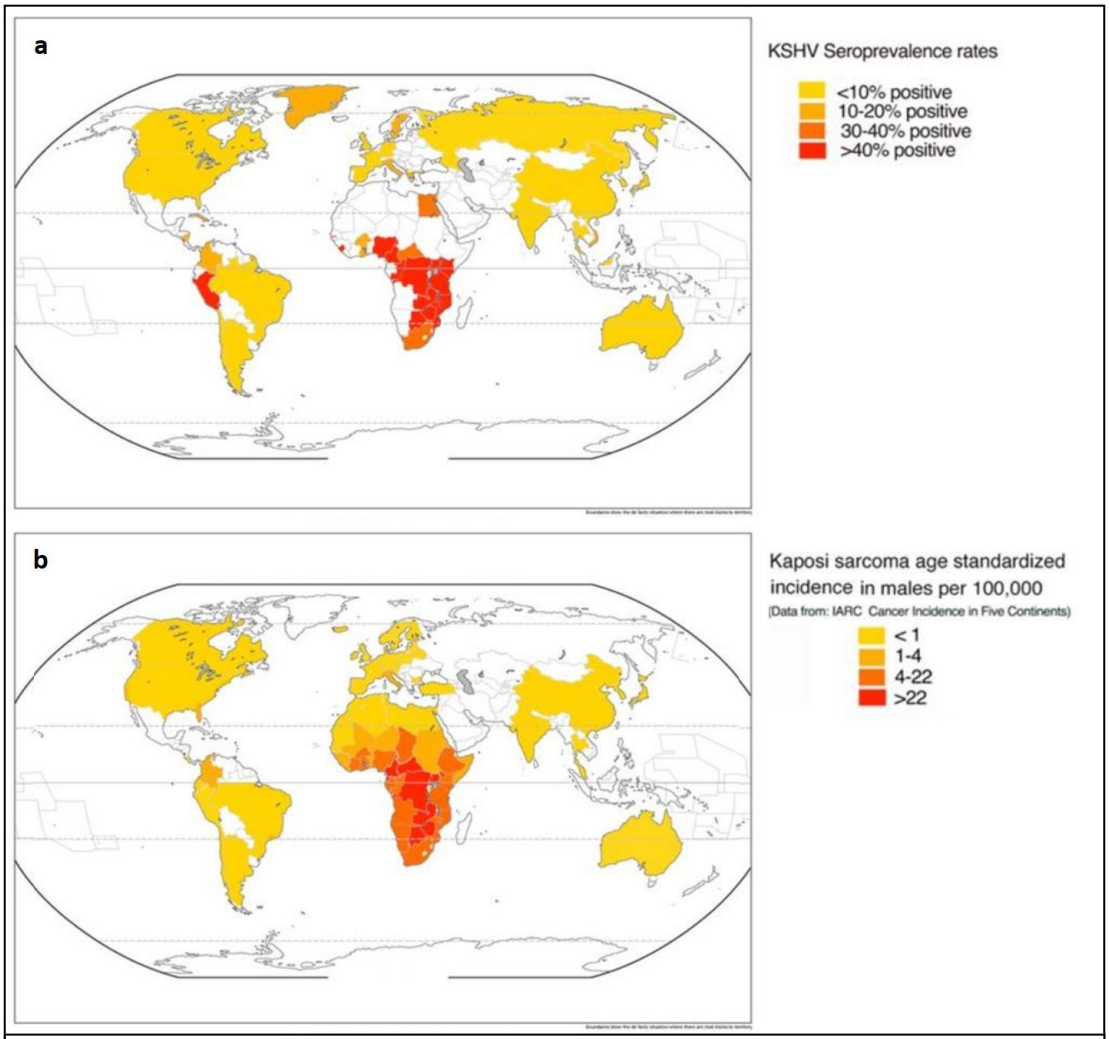

Figure 2. Geographical seroprevalence of KSHV and prevalence of KS. a. Seroprevalence rates in general population compiled from averages of multiple studies and **b.** The standardized incidence of KS in males (obtained from the International Agency for Research on Cancer WHO publication: Cancer Incidence in Five Continents) Reproduced with the permission of the publisher from (Mesri, Cesarman, and Boshoff 2010).

2.2.3 Transmission of KSHV

KSHV viral reservoirs are maintained in the cells of oral cavity, from where new viral particles are produced and significant amounts of virions are secreted in the saliva of infected individuals (Vieira et al. 1997; Pauk et al. 2000). There are several indications

of KSHV shedding in saliva that suggest it as a natural source of transmission. In endemic KS areas, transmission from mother or other intimate care-taker to children before puberty provides evidence of non-sexual horizontal transmission, probably occurring through saliva (Mayama et al. 1998). In addition to childhood infections, KSHV is documented to be transmitted through blood transfusion and organ transplantation (Martin 2007). Nevertheless, especially sexual transmission between homosexuals with multiple sexual partners, is linked to higher risk of persistent infection and KS development than through heterosexual activities or parenteral transmission of the virus (Martin et al. 1998; Blackbourn et al. 1999). Even though KSHV infection is common among homosexual men, it is not increased among intravenous drug users, suggesting that transmission through blood inoculation is not a prominent route (Zhang et al. 1998).

2.2.4 Life cycle of KSHV

The hallmark of Herpesviridae is to establish lifelong infection in the host, displaying biphasic life cycle of quiescent latency and active lytic replication (Renne, Lagunoff, et al. 1996; Miller et al. 1997). In latency, the virus stays dormant within the host, expressing only a few genes and no viral particles are produced (Roizman and Pellet 2001). Turning off "unnecessary" viral proteins during latency serves as a strategy for the viruses to maintain persistent infection by avoiding host immune surveillance. Lifelong infection requires successful establishment and maintenance of latency as a viral episome in the nucleus of the host cell (Ballestas, Chatis, and Kaye 1999). However, reactivation from latency to lytic replication is necessary for the production of new viral particles to disseminate within the host and to spread by transmission to other individuals. The switch to lytic replication cycle initiates the expression of all the viral genes in a temporally organized cascade resulting in the release of progeny virions and inflammatory cytokines inducing host responses (Ensoli and Sturzl 1998; Renne, Lagunoff, et al. 1996).

Characteristics of the herpesvirus family, including KSHV, is the very large size of the double-stranded DNA (dsDNA) genome and architecture of the virion structure consisting of icosahedral protein capsid, tegument proteins and lipid envelope (Chang et al. 1994; Wu et al. 2000; Roizman and Pellet 2001). The 165kb dsDNA genome of KSHV

circulates to form a covalently closed episome including 140kb of coding information and 87 open reading frames (ORFs) (Russo et al. 1996; Renne, Lagunoff, et al. 1996). Some of the genes are apparently hijacked from the host cells, some are conserved among herpesviruses and some are unique for KSHV (Moore and Chang 2001). KSHV genes can be classified into four distinct kinetic stages, from latency to lytic viral transcription in an orderly cascade: (i) latent genes are expressed during the quiescent latency; (ii) immediate early (IE) genes do not require prior viral protein synthesis for their expression; (iii) early (E) genes are expressed independent of viral DNA synthesis and (iv) late (L) genes whose expression is dependent on viral DNA synthesis (Roizman and Pellet 2001).

2.2.4.1 Primary infection

Primary KSHV infection is usually asymptomatic or symptoms are mild. However, in individuals with immunosuppression, the primary symptoms may be more notable (Luppi et al. 2002). *In vitro,* KSHV has been shown to have a broad cellular tropism infecting B-cells, fibroblasts, macrophages, epithelial and primary endothelial cells (Akula et al. 2001). When reaching the target cell, virus attaches to the cell surface molecule heparan sulfate (HS), which is a ubiquitous linear polysaccharide (Wang et al. 2001; Birkmann et al. 2001). The efficient entry into the host is achieved by fusion of the envelope with the host cell membrane (Sieczkarski and Whittaker 2002).

After the entry into the host cell, the viral capsid is trafficked through the cytoplasm to deliver KSHV viral genome into the nucleus, where it is released from the capsid and immediately circularized to become an episome (Chandran and Hutt-Fletcher 2007). In the nucleus of the infected cell, the virus initiates the lytic replication cycle and initiates transcription of lytic genes. Replication and transcription activator (RTA) has been identified through kinetic studies as an IE gene product, encoded by ORF50, the earliest viral gene to be expressed (Sun et al. 1999). Functional experiments have shown the necessary and sufficient role of RTA in initiation of KSHV lytic replication (Sun et al. 1998). However, it has been shown that during the *de novo* infection only a limited number of lytic genes are expressed, most of which decrease rapidly during the course of

infection (Krishnan et al. 2004). This initial lytic activation, also indicated as the lytic burst, does not complete the lytic cascade to produce progeny virions but rather amplifies the viral episome and mediates immune modulation and anti-apoptotic functions. Indeed, concurrently with the lytic burst, expression of latent genes was shown to increase following the primary infection and were maintained during the course of infection, whereas expression of lytic genes subsequently decreased, indicating establishment of latency (Krishnan et al. 2004).

2.2.4.2 Latency establishment

During latency, KSHV genome persist as multiple copies of extrachromosomal episomes in the host cell nucleus. Viral gene expression is restricted to a few latent genes and miRNAs, which do not drive the production of progeny virions (Roizman and Pellet 2001). Covalently closed circular episomes are hitchhiked to host chromatin by the latency-associated nuclear antigen (LANA), a multifunctional latent viral protein. To persist in the proliferating cells, episomes must replicate prior to cell division to maintain sufficient copy number within the new host cells. Once the host cell undergoes cell division, the latent episome relies on the host cell machinery and is replicated in synchrony with the cellular DNA (Ballestas, Chatis, and Kaye 1999). Also, efficient segregation of episomes to nuclei of the daughter cells after mitosis is necessary to avoid cytoplasmic degradation of the episomal DNA.

ORF73 has been identified as the gene encoding LANA (Kedes et al. 1997), and LANA plays a critical role in maintaining the viral episome (Ballestas, Chatis, and Kaye 1999). LANA mediates the replication of KSHV DNA and the chromatin-binding domains of LANA tether the viral episome to mitotic chromosomes and help to segregate the replicated episomes to progeny nuclei (Piolot et al. 2001; Ballestas and Kaye 2001; Grant et al. 2018) To achieve the crucial functions of episome persistence, LANA associates with different host cell proteins involved in DNA replication, transcriptional regulation and growth control (Friborg et al. 1999; Radkov, Kellam, and Boshoff 2000). However, latently infected cells tend to lose episomes upon culturing indicating that the function of LANA is not always sufficient to stably maintain latency (Grundhoff and

Ganem 2004). Especially rapidly dividing cells and *in vitro* cultured explanted KS spindle cells lose viral genomes after only a few passages (Dictor et al. 1996).

2.2.4.3 Reactivation from latency

Latency can be disrupted upon appropriate induction or spontaneous reactivation of KSHV, displaying the nature of recurrent infectious cycle of herpesviruses. The reactivation of KSHV is triggered by the expression of the IE gene RTA, indicating its crucial role in the switch between latency and lytic replication (Zhu, Cusano, and Yuan 1999). RTA is necessary and sufficient to activate the transcription of all the downstream IE, E and L viral genes (Sun et al. 1998).

In KSHV-infected cells, the interplay between RTA and LANA controls the switch from latency to lytic cycle. The earliest lytic gene RTA activates LANA to initiate establishment of latency. Reciprocally, LANA expression downregulates RTA by repressing its promoter (Lan et al. 2005). Epigenetic DNA methylation of the ORF50 promoter facilitates the establishment and maintenance of latency in vivo (Chen et al. 2001), thus keeping RTA at bay from further initiating lytic replication cascade. Also, viral non-coding polyadenylated nuclear (PAN) RNA is a regulatory transcript that mediates chromatin remodeling and targeting the repressed KSHV gene expression (Rossetto and Pari 2014; Aneja and Yuan 2017). Consequently, transcription of RTA is activated by epigenetic regulation, having a role in the reactivation of KSHV.

Spontaneous reactivation of KSHV occurs periodically in the latently infected cells, and several mechanisms have been shown to trigger reactivation. In addition to above-mentioned immunosuppression due to organ transplantation or AIDS, also other viral co-infections and unbalanced inflammatory cytokines (Mercader et al. 2000; Vieira et al. 2001), hypoxia (Davis et al. 2001), oxidative stress (Ye et al. 2011), and certain chemicals have been shown to reactivate KSHV. Inducing chemical agents are used in the *in vitro* studies to achieve efficient reactivation. For example, sodium butyrate (NaB) is a histone deacetylase (HDAc) and releases the epigenetic repression of RTA by chromatin modifications, boosting the induction of KSHV lytic expression (Miller et al. 1997).

2.2.4.4 Productive lytic replication

The lytic cycle is necessary for the virus to propagate and release infectious viral particles. The viral genome provides instructions for the production and assembly of progeny viral particles (Figure 3) and the host cell provides the machinery and precursor molecules to produce needed viral proteins and nucleic acids.

RTA initiates the transcription of several downstream genes (Jenkins and Hoffman 2000). ORF45 is expressed as an IE gene in the beginning of the lytic cycle and its synthesis remains high through the late stages of the lytic replication (Zhu, Cusano, and Yuan 1999). Mostly cytoplasmic ORF45 protein is a highly phosphorylated component of the KSHV tegument, and significant amounts can be located in the encapsidated virions (Zhu et al. 2005). RTA also selectively activates promoter of E gene encoding a nuclear protein ORF57, also known as the mRNA transport and accumulation protein (MTA), which is multifunctional and conserved protein among herpesviruses, facilitating lytic replication (Bello et al. 1999; Lukac et al. 2001). ORF57 has been shown to have an essential role in lytic replication, as ORF57-null recombinant KSHV was unable to efficiently express several other lytic genes or produce virions (Han and Swaminathan 2006). ORF57 has been shown to facilitate translation and nuclear export of viral intronless mRNAs by recruiting cellular mRNA processing pathways, thereby enhancing lytic protein expression and virion production (Boyne, Jackson, and Whitehouse 2010; Schumann, Baquero-Perez, and Whitehouse 2016). One of the genes unique to KSHV (with no known sequence similarity to any other pathogen), K8.1, has been identified as a L gene through kinetic studies (Chandran et al. 1998). K8.1 encoding a structural envelope glycoprotein incorporated in the KSHV virion has an essential role in the production of new viral particles and it is acquired on the virion surface during budding from the plasma membrane (Birkmann et al. 2001). The L genes encode primarily viral structural proteins and reach the peak rates of protein synthesis after the onset of DNA replication (Jenkins and Hoffman 2000). Viral lytic replication amplifies the viral DNA through a rolling circle mechanism to replace the segregated episomes and to maintain the population of infected cells (Lukac and Yuan 2007).

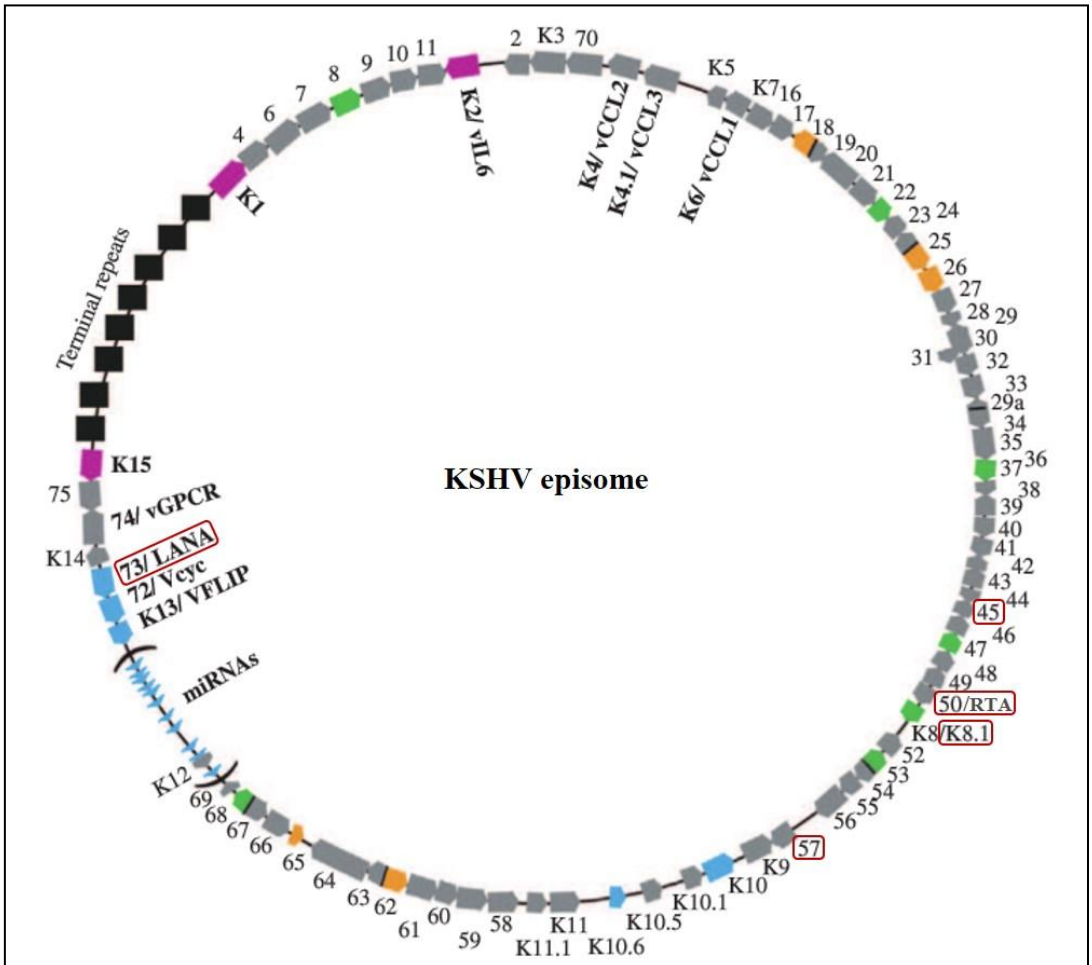

Figure 3. Map of the KSHV genome organization. The direction of the transcription is shown with the arrows, ORFs are indicated with numbers, genes unique for KSHV indicated with K. Genes highly expressed during latency are depicted with blue, and with purple those which are expressed in low levels with increase after switch to lytic replication. Lytic genes transcripted are depicted with grey and of those necessary for virion production are the capsid and envelope genes depicted with orange and green, respectively. Genes discussed in the text are highlighted with red. Reproduced with the permission of the publisher from (Gramolelli and Schulz 2015).

All in all, during the productive lytic replication the newly synthesized viral dsDNA is incorporated into the preformed intranuclear capsid, which proceeds to cytoplasm to acquire the tegument structure. Final envelopment occurs by budding into Golgi-derived vesicles where the amplified progeny virions are assembled and released from the cells by fusing with the cell plasma membrane (Mettenleiter, Klupp, and Granzow 2009). The release of multiple encapsidated virus particles induces a cytopathic effect and is extremely lethal to the host cell. Released mature virions disseminate within the host to infect susceptible cells and can be transmitted to new hosts (Jenkins and Hoffman 2000).

2.2.5 The contribution of KSHV latent and lytic phases to KS tumorigenesis

The development of KS is initiated by the latent KSHV infection of the host cells, and it is considered that the latency program expressing only a few viral genes drives the tumorigenesis. In addition to the crucial role in the latent episome persistence (Ballestas, Chatis, and Kaye 1999), LANA contributes to KSHV-mediated oncogenesis by associating with a variety of host cell proteins of DNA replication, transcriptional regulation and growth control. Acting as a transcriptional co-factor, LANA has been shown to promote cell proliferation and survival by repressing or modulating tumor suppressor protein retinoblastoma (pRb) and p53 signaling pathways (Friborg et al. 1999; Radkov, Kellam, and Boshoff 2000; Sarek and Ojala 2007). The latency locus also encodes several other proteins; Kaposin, viral cyclin (v-cyclin), viral FLICE-inhibitory protein (vFLIP), and the viral-encoded microRNAs, which have been shown to contribute to KS tumorigenesis by promoting cell growth and inhibiting apoptosis (Muralidhar et al. 1998; Verschuren et al. 2002; Sturzl et al. 1999; Sun, Zachariah, and Chaudhary 2003). Even though the latent KSHV gene expression supports the tumorigenesis of KS, it is not sufficient to sustain the development of the neoplasm.

Most of the SC from KS lesions predominantly display latent KSHV infection, with only a minority of the cells undergoing spontaneous lytic reactivation (Figure 4) resulting in the expression of lytic viral proteins and virion production (Staskus et al. 1997; Sun et al. 1999; Dupin et al. 1999). Low and declining antibody titers in individuals with functional immune surveillance also reflect the low rate of spontaneous reactivation (Martro et al. 2004). *In vitro*, infected SC tend to lose the viral episomes upon rounds of cell divisions. The role of lytic reactivation resulting in the release of infectious progeny is to counteract this inefficient episome maintenance by providing new viral particles that can infect new cells. The recruitment of newly infected cells in the tumors is necessary for sustaining the population of latent cells with viral genome, suggesting that lytic replication also contributes to KS tumorigenesis (Grundhoff and Ganem 2004). Furthermore, evidence for this notion is provided by the observed decrease in the incidence of AIDS-KS and regression of existing KS lesions in patients treated with the anti-herpesviral drug ganciclovir (Martin et al. 1999). Ganciclovir blocks lytic replication

by binding specifically to the viral DNA polymerase expressed during the lytic phase, having no effect on latent cells. The ability of ganciclovir to inhibit KS progression indicates that KSHV lytic replication is necessary for KS to develop and sustain. Moreover, a study with nude mice showed that latent cells were not able to induce tumor formation, whereas ectopic expression of viral G protein-coupled receptor (vGPCR), expressed only during lytic replication, was able to give rise to tumors resembling human KS (Montaner et al. 2003; Montaner et al. 2006).

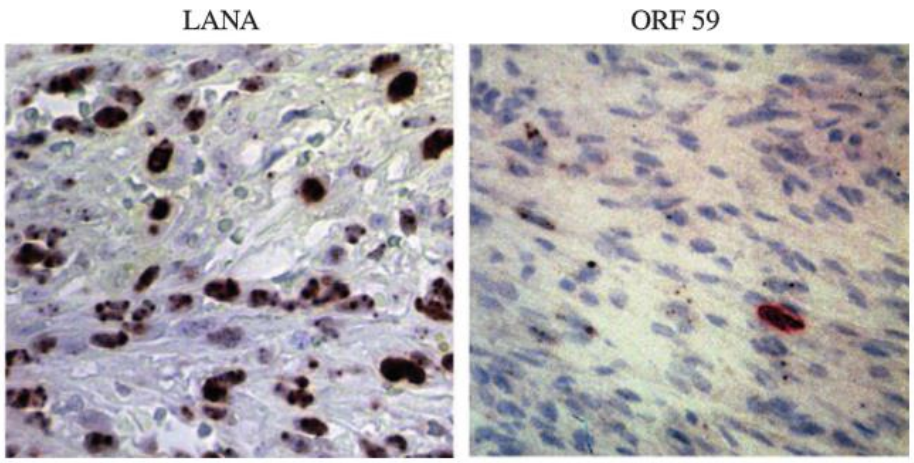

Figure 4. Expression of latent LANA and an early lytic protein ORF59 of KSHV in KS lesion biopsy. KS biopsy stained with specific antibody against LANA (left) and ORF59 (right). Majority of the cells are in latency, with only a few spontaneously reactivating to lytic replication, indicated with the early lytic protein ORF59. Reproduced with the permission of the publisher from (Schulz 2006).

However, only a fraction of infected cells in KS lesions reactivate to produce KSHV-encoded lytic oncogenes and the survival of the lytic cells is only transient as the completion of the lytic cycle and release of infectious virions will kill the host cell. The common hypothesis is that lytically infected cells contribute to KS tumor development also by producing and activating signaling molecules which promote inflammation, modulate cell migration and stimulate angiogenesis in the KS lesions in a paracrine manner (Jochmann et al. 2012). Indeed, vGPCR enhances cell proliferation, angiogenesis and invasion, both in an autocrine and paracrine fashion (Bais et al. 1998; Arvanitakis et al. 1997; Jham and Montaner 2010). Similarly, a viral transmembrane protein K1 induces

the secretion of cellular inflammatory cytokines, growth factors and matrix metalloproteinase-9 (MMP-9) in ECs (Wang, Wakisaka, et al. 2004). This so called paracrine effect, indicating secretion of cytokines and chemokines either encoded by the virus or induced by the expression of lytic genes, is a very important driver of KSHV pathogenesis (Jochmann et al. 2012).

Generally, in immunocompetent individuals the sporadic spontaneous reactivation of KSHV is successfully cleared by a functional immune system (Simpson et al. 1996). The infection is under control, and majority of the infected cells avoid provoking host responses and elimination. As the virus is dependent on the host to survive and reproduce, it is not convenient for viruses to exhaust their residence. Disruption of the balance between viral infection and host immunity leads to agitated reactivation and increased lytic replication (Aneja and Yuan 2017). Consequently, more KS promoting lytic oncogenes are encoded and cytokines and chemokines secreted, in addition to enhanced production of new viral particles to infect nearby cells. In case of immunodeficient individuals, the CD4+ T-cell count and the host immune response is not sufficient to suppress the infection, and KSHV thrives uncontrollably (O'Connor and T-Scadden 2004). Accordingly, AIDS patients are more susceptible to develop KS.

In conclusion, KSHV latency drives oncogenesis of infected SC in KS lesions by inducing cell proliferation and reprogramming, and preventing apoptosis of infected cells, whereas KSHV lytic genes take part in the inflammatory and angiogenic phenotype of KS lesions. In addition to the crucial role in maintaining a population of infected cells, lytic replication triggers host signaling cascades leading to recruitment of inflammatory cells and secretion of cytokines and pro-angiogenic growth factors. Thus, both latent and lytic viral cycles contribute significantly to the tumorigenic microenvironment and phenotype of infected SC and to the oncogenesis of KS.

2.3 KSHV-mediated endothelial cell reprogramming

2.3.1 Angiogenesis and lymphangiogenesis

The circulatory system consists of blood vessels and lymphatic vessels interconnected to form a complex and functional network able to deliver oxygen, nutrients and hormones throughout the body and to regulate fluid homeostasis and metabolic waste products. In addition, the lymphatic vascular system is essential for absorption of dietary fat and immune cell trafficking as part of immune surveillance (Witte et al. 2001; Alitalo, Tammela, and Petrova 2005). The formation and growth of new blood and lymphatic vasculature, termed angiogenesis and lymphangiogenesis respectively, occurs mainly early during embryogenesis. Additionally, the plasticity of vasculature is retained in the adulthood, allowing tissue repair and remodeling, but also aberrant angiogenesis and lymphangiogenesis in pathological processes, such as in KS, may occur (Adams and Alitalo 2007).

During angio- and lymphangiogenesis, vessels are formed by squamous ECs lining the interior surface of the new vessel as a continuous layer. Vascular smooth muscle cells and pericytes form the stabilizing outer layer. Endothelial precursor cells differentiate from mesodermal cells in embryogenesis and aggregate to form primary vascular plexus, which remodels through angiogenesis in a hierarchal vascular system. Maturation of blood endothelial cells (BECs) is executed to acquire tissue-specific specialization of vascular endothelium as a circulatory system (Gale and Yancopoulos 1999; Alitalo, Tammela, and Petrova 2005). After the development of the cardiovascular system, subpopulation of ECs commit to lymphatic lineage and sprout from the embryonic cardinal veins to form the lymphatic system. (Wigle and Oliver 1999). Differentiated lymphatic endothelial cells (LECs) are distinct from BECs, by virtue of differential expression of specific transcripts. Blood and lymphatic vessel morphogenic events in both the normal development and in pathological remodeling are mediated by vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs). VEGFR-1 and -2 expressions are specific for BECs whereas VEGFR-3 is predominantly expressed in LECs (Ferrara, Gerber, and LeCouter 2003).

2.3.2 Transcription factor Prox1

The prospero-related homeobox gene 1 (*Prox1*) is essential in acquisition of the LEC identity, contributing to the initial commitment during embryonic development. Prox1 regulates LECs to differentiate from BECs in lymphangiogenesis (Yoo et al. 2012; Cancian, Hansen, and Boshoff 2013). Accordingly, Prox1 null mice embryos fail to differentiate the lymphatic vasculature, resulting in embryonic death (Wigle and Oliver 1999; Wigle et al. 2002). *In vitro*, Prox1 has been shown to convert transcriptional program of cultured BECs towards the LEC phenotype, to adopt relevant function of lymphatic vasculature by upregulating LEC -specific gene expression, such as VEGFR-3 (*FLT4*). In addition, several markers associated with the BEC phenotype have been shown to be suppressed by overexpression of Prox1, further promoting lymphatic endothelial phenotype development (Petrova et al. 2002; Hong et al. 2002). Eventually, in the differentiated BECs Prox1 is absent, whereas in LECs it is highly expressed, further supporting the notion that Prox1 is a master regulator of LEC differentiation and fate (Hong et al. 2004).

Transcription factor Prox1 is localized on chromosome 1q32.2-q32.3 and it is composed of five exons and four introns, and codes for a protein with a predicted molecular mass of 82.3kDa (Elsir et al. 2012). The Homeo-prospero domain is highly conserved among vertebrates and Prox1 is the mammalian homolog of the *Drosophila* prospero gene, which specifies the cell fate in the insect neuronal system (Doe et al. 1991). Likewise, Prox1 in humans has emerged as a key regulatory protein in the development of various organs, in addition to the lymphatic system. Binding of Prox1 to DNA allows transcriptional regulation of other genes required for the development of lens (Wigle et al. 1999), liver (Sosa-Pineda, Wigle, and Oliver 2000), pancreas (Burke and Oliver 2002), brain (Lavado and Oliver 2007), ear (Kirjavainen et al. 2008), and heart (Risebro et al. 2009). Furthermore, altered expression and function of Prox1 is associated with a number of human cancers. In different cancers, Prox1 showed both tumor suppressor and oncogenic properties depending on the context (Elsir et al. 2012).

2.3.3 Prox1 in KS

KSHV infection of ECs activates a developmental genetic program specifying the LEC fate by altered Prox1 expression. Infection of differentiated BECs leads to lymphatic reprogramming by induction of LEC specific genes, including Prox1, and downregulation of blood vascular genes to acquire a partial LEC phenotype (Hong et al. 2004). Essentially, this leads to BEC forward reprogramming towards a more differentiated lymphatic phenotype, as Prox1 regulates BECs to differentiate into LECs in embryonic lymphangiogenesis. In a similar manner, KSHV-infected LECs shift towards blood vascular-like phenotype by downregulation of Prox1 (Wang, Trotter, et al. 2004). Infected LECs can be implied to undergo reverse reprogramming to a less differentiated blood vascular-like phenotype. The incomplete KSHV-mediated differentiation events of BECs and LECs according to expression of Prox1 are schematically summarized in Figure 5.

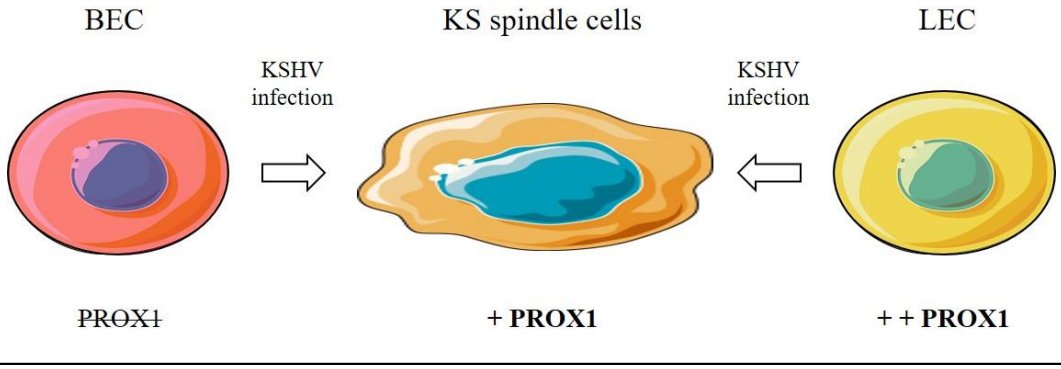

Figure 5. KSHV-mediated host cell fate reprogramming towards KS spindle cell by altered transcriptional expression of Prox1. Differentiated BECs do not express Prox1, whereas differentiated mature LECs have high expression of Prox1. Upon infection, distinct endothelial lineages acquire mixed identity of KS spindle cells through endothelial reprogramming, as Prox1 expression is upregulated in BECs and downregulated in LECs.

Evidence supports the idea that Prox1 has an important role in mediating the effects of KSHV on bidirectional endothelial reprogramming resulting in the mixed identity (Yoo et al. 2012). Recent studies have provided insights to the molecular mechanisms of KSHV-induced changes of Prox1 expression in ECs. Several host signaling pathways and transcriptional factors have been identified to regulate Prox1, and multiple viral genes to deregulate the expression of Prox1 (Emuss et al. 2009; Yoo et al.

2010; Cheng et al. 2011; Yoo et al. 2012; Chang and Ganem 2013). Findings on KSHV-mediated EC reprogramming elucidates the importance of Prox1 in KS tumorigenesis. The expression of Prox1 was found to increase from early stage KS to advanced nodular stage, where the majority (93.3%) of analysed neoplastic SCs were positive for Prox1, further suggesting the involvement of Prox1 in the pathogenesis of KS (Benevenuto de Andrade et al. 2014).

2.3.4 Endothelial cell reprogramming towards KS spindle cell

SC, the principal proliferating cell in advanced KS lesions, was first named as such in the 1883 by German pathologist Köbner (Gessain and Duprez 2005). KSHV is latent in up to 90% of the spindle cells in the nodular advanced KS lesion and the productive virus replication is restricted to only a few spontaneously reactivating cells (Staskus et al. 1997; Boshoff, Schulz, et al. 1995). KSHV infection of ECs causes rearrangement of the actin cytoskeleton producing elongated spindle-like morphology (Grossmann et al. 2006), changes in the glucose metabolism (Delgado et al. 2010), and proliferative advantage by altered growth rate and gene expression (Wang, Trotter, et al. 2004; Cheng, Pekkonen, and Ojala 2012). KSHV-mediated acquisition of SC morphology is illustrated in Figure 6. Furthermore, SCs display other tumorigenic properties such as increased angiogenic and migratory capacities (Wang and Damania 2008; Cheng et al. 2011; Ojala and Schulz 2014). All of these characteristics of SC contribute to the diffusion of KS, highlighting the crucial role of KSHV-mediated reprogramming of ECs as a driver of the KS pathogenesis.

BECs and LECs display morphological and functional similarities but are yet distinct from each other having significant plasticity and heterogeneity upon pathological reprogramming (Ferrara 1999; Adams and Alitalo 2007). KSHV infection modifies EC identity by manipulating the expression of endothelial-specific factors, among others Prox1, thus inducing a deep reprogramming towards a more invasive SC phenotype.

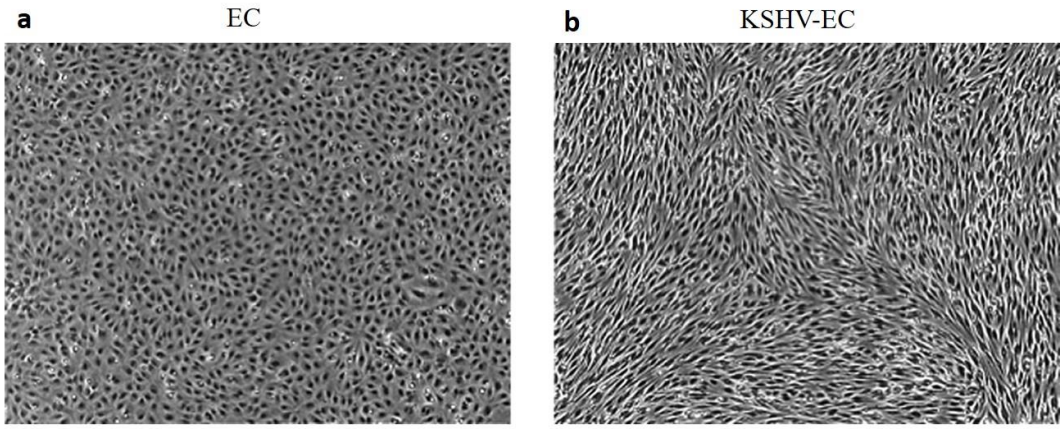

Figure 6. Latent infection by KSHV causes endothelial cells to acquire spindle morphology. a, b. Human umbilical vein endothelial cells (HUVEC) prior (**a**) and after (**b**) infection with KSHV in culture. Reproduced with the permission of the publisher from (Ganem 2006).

The origin of SC has been under debate as the marker expression is highly heterogeneous. SC are believed to be of endothelial origin as they express several endothelial markers (Ensoli et al. 2001; Boshoff, Schulz, et al. 1995), however, they also display features of other cell lineages such as fibroblasts, macrophages and smooth muscle cells (Weich et al. 1991; Simonart et al. 2002). Whether spindle cells are originally derived from infected BECs or LECs is still under investigation although several studies argue for lymphatic endothelial lineage origin. *In vitro* studies have shown infected LECs to resemble the elongated morphology of KS spindle cells more distinctly than BECs do. Also, LECs support KSHV infection more efficiently harboring higher viral copy numbers compared to BECs, suggesting that LECs represents a preferred target of infection (Wang, Trotter, et al. 2004). Accordingly, KS occurs more frequently on the skin, in lymph nodes and mucosa abundant with LECs, but not in organs devoid of lymphatic vessels (Pantanowitz and Dezube 2008). Furthermore, gene expression signature of spindle cells resembles that of LECs with substantial expression of several LEC markers, including VEGFR-3 and podoplanin, when compared to the BEC specific markers (Weninger et al. 1999; Wang, Trotter, et al. 2004).

However, the gene expression profiles of infected LECs and BECs show that KSHV induces transcriptional reprogramming of both cell types (Wang, Trotter, et al. 2004; Hong et al. 2004). The transcriptional drift in both LECs and BECs results in

mixed identity, somewhere in between the two phenotypes without reaching complete differentiation of one cell type towards the other (Yoo et al. 2012). Dysregulated transcriptional program explains the pleiotropy of marker expression in SC and the heterogeneity of KS lesions, contributing to Kaposi's sarcoma tumorigenesis (Gramolelli and Ojala 2017) (Figure 7).

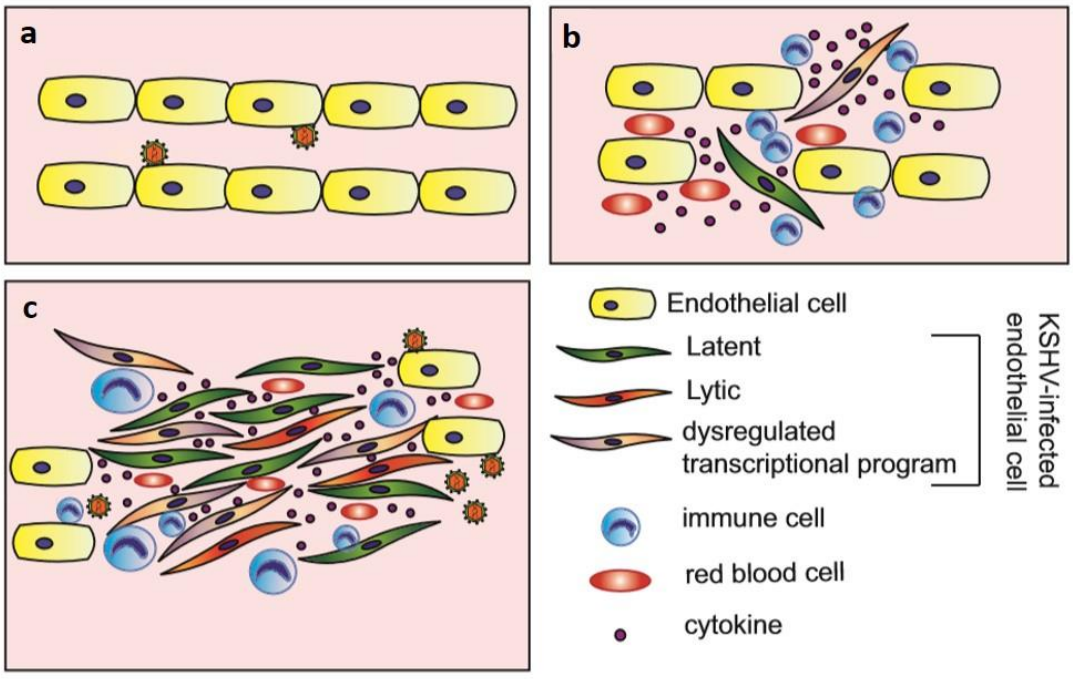

Figure 7. Schematic model of a KS lesion a-c. a. KSHV virions infect ECs. **b.** Early KS lesion with infected ECs reprogrammed towards SC phenotype producing cytokines attracting immune cells and inducing extravasation of red blood cells through angiogenesis. **c.** Late KS lesion with majority of SCs in latent phase and subset expressing oncogenic viral proteins and producing progeny virions allowing continuous reinfection of ECs attracted to the tumor. Reproduced with the permission of the publisher from (Gramolelli and Ojala 2017).

3 Aim of the study

The switch from latency to productive lytic replication is an essential step in the pathogenesis of KS. Previous studies support the importance of lytic replication in the development and progression of KS (Martin et al. 1999; Grundhoff and Ganem 2004). Furthermore, lytic reactivation initiates the production of both viral and cellular inflammatory and angiogenic cytokines in the tumor microenvironment (Ensoli and Sturzl 1998). The expression of Prox1, the master regulator of lymphatic endothelial cell fate, is altered upon KSHV infection of ECs (Hong et al. 2004). Prox1 has an important role in the KSHV-mediated EC reprogramming towards the SC phenotype, which in turn supports the generation of a pro-angiogenic and inflammatory microenvironment, thus contributing to KS tumorigenesis (Yoo et al. 2012; Gramolelli and Ojala 2017). However, the role of Prox1 in lytic reactivation has not been assessed. Preliminary studies in our group carried out in KSHV-infected ECs indicate that Prox1 depletion leads to a decrease in lytic reactivation, thus suggesting a positive role for Prox1 in KSHV reactivation.

The aim of the thesis was to elucidate the role of Prox1 in KSHV reactivation from latency and the effect of Prox1 in lytic replication. My aim was to investigate the effect of ectopic expression of Prox1 on viral reactivation and lytic replication efficacy, and to measure the effect on production of progeny viral particles. The effort of gaining deeper understanding of the role of Prox1 in KSHV life cycle can potentially open new translational leads to more efficient therapeutic strategies to tackle KSHV infection and KS progression.

4 Materials and methods

4.1 Cell lines

iSLK.219 rKSHV is a RTA -inducible SLK cell line, stably infected with a recombinant KSHV.219. SLK cells are originally derived from an oral KS lesion biopsy of a HIV-negative renal transplant KS patient (Herndier et al. 1994; Siegal et al. 1990). These cells were reported to be uninfected cells of endothelial origin and when implanted in mice they formed highly vascularized tumors reminiscent of KS (Dr. J. A. Levy United States (Herndier et al. 1994) However, more recently when the authenticity of SLK cells was tested, it was shown that these cells have been cross-contaminated relatively early in their passage and are of epithelial-cell origin, indistinguishable from the clear-cell renal-cell carcinoma cell line Caki-1. Therefore, the SLK cell line is not a suitable model for endothelial tumor cell biology. However, these cells are fully permissive for the KSHV latent and lytic replicative cycles and retain their utility for the study of KSHV gene expression (Sturzl et al. 2013).

SLK were engineered to express RTA under a TET (tetracycline) responsive promoter, meaning that RTA promoter is activated after induction with doxycycline (iSLK) (Myoung and Ganem 2011). Cells were latently infected with recombinant KSHV.219 virus (iSLK.219) expressing green fluorescent protein (GFP) by the constitutively active human elongation factor 1-α (EF-1α) promoter and red fluorescent protein (RFP) under the control of RTA-responsive polyadenylated nuclear (PAN) promoter, expressed only during lytic replication (Vieira and O'Hearn 2004). In addition, *pac*-gene under control of the Rous sarcoma virus (RSV) promoter is inserted to give puromycin-resistance as an selection marker (Myoung and Ganem 2011). This cell line is a useful and well-characterized model to study KSHV latency and viral replication due to the tight latent program and to the efficient and synchronous induction of lytic reactivation by the Doxycycline-dependent induction of the RTA gene. Furthermore, latent and lytic expression can be monitored by the expression of GFP and RFP, respectively. A schematic diagram of the recombinant cell line iSLK.219 is presented in Figure 8.

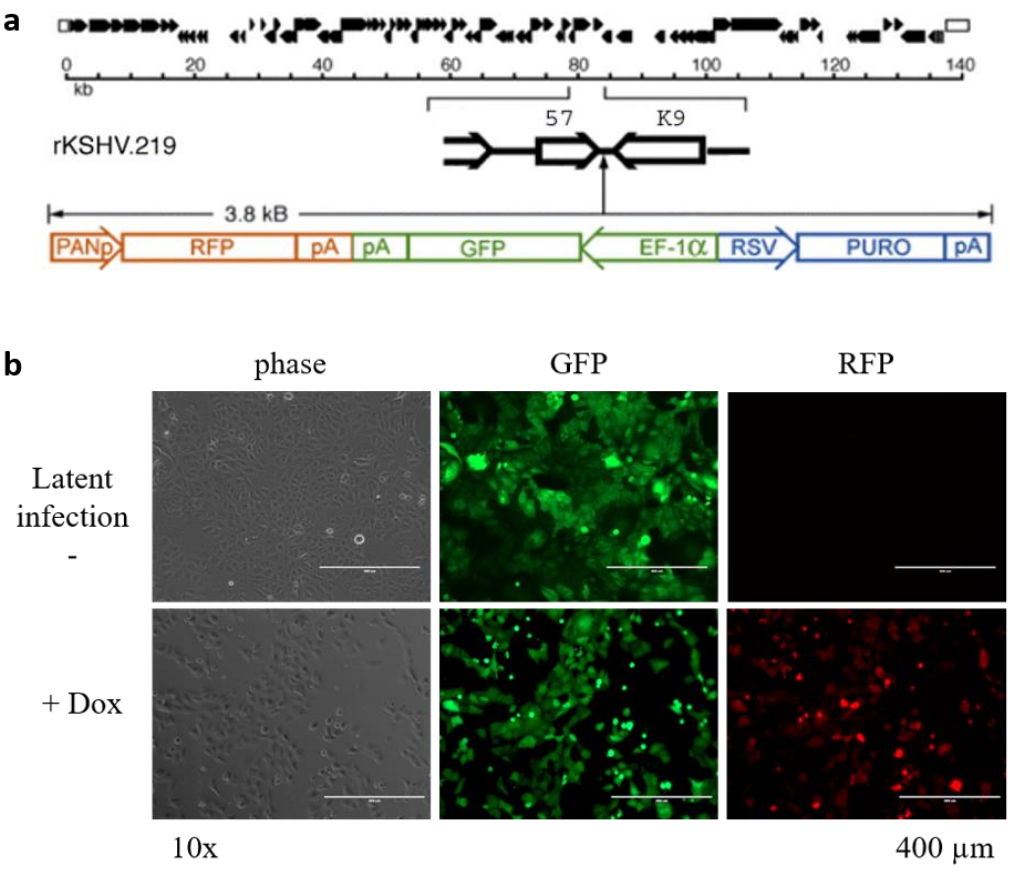

Figure 8. Schematic diagram of the recombinant cell line iSLK rKSHV.219 a, b.
a. Schematic diagram of KSHV.219 genome with magnified structure of the inserted construct between ORF57 and K9 at non-coding area. The inserted cassette includes the RFP gene under PAN promoter, GFP gene under EF1-α promoter, and puromycin resistance gene under RSV promoter. The numbers indicate kilobases and direction of the transcription is shown by the arrows. Reproduced with the permission of the publisher from (Vieira and O'Hearn 2004).
b. Phase and fluorescent microscope (10x) images of iSLK.219 cells upon latent infection expressing GFP or + Dox indicating induced lytic reactivation expressing RFP.

Human osteosarcoma cell line U2OS was used as a naïve, uninfected cell line to study KSHV infection kinetics and efficacy of infection in the virus release assays. This cell line is highly susceptible to KSHV infection. Human embryonic kidney cell line HEK 293FT (National Cancer Institute, LCO technical files) is a fast-growing variant of the parent cell line (HEK293T) and was used for generating high-titer lentivirus. TREx-RTA BCBL-1, a KSHV-positive B cell line from a PEL patient (Renne, Zhong, et al. 1996) with a Dox-inducible RTA construct, was used to produce wtKSHV for the infection kinetics assay.

BCBL1 cells were grown in RPMI 1640 medium and iSLK.219 rKSHV, U2OS and HEK 293FT cells were grown in Dulbecco modified Eagle´s medium (DMEM), both purchased from Lonza and supplemented with 10% Foetal Calf serum (FCS) and 1% L-Glutamine. iSLK.219 cells were cultured with puromycin 1µg/mL (Sigma P8833) for selection of infected cells. In addition, G418 solution (Roche) in dilution 1:100 and hygromycin (Thermo Fisher Scientific) in dilution 1:100 was added to the iSLK.219 cells to allow the activation of RTA promoter. All cells were propagated at standard conditions (37°C, 16% O_2, 79% N_2, 5% CO_2) using Heraeus Hera cell 150 incubator (Thermo Electron Corporation).

4.2 Antibodies

Primary antibodies used in Western blotting, immunofluorescence staining or in immunoprecipitation of this study are listed in Table 1.

Table 1. Primary antibodies.

Antibody against/ stain	description	source/reference	Working dilution	Used in
β -actin	Mouse antibody	Santa Cruz (sc-47778)	1:500	WB
GFP	Rabbit antibody	A gift from Ari Helenius	1:2500	IF
K8.1	Mouse antibody	Santa Cruz (sc-65446)	1:200 1:1000	IF WB
LANA	Rat antibody	Abcam (ab4103)	1:200 1:1000	IF WB
normal mouse IgG	Mouse antibody	Santa Cruz (sc-2025)	1:40	IP
MYC	Mouse antibody	Cell Signaling (9B11)	1:20	IP
ORF45	Mouse antibody	Santa Cruz (sc-53883)	1:200 1:1000	IF WB
ORF50/RTA	Rabbit antibody	A gift from Carolina A. Arias	1:1000	WB
ORF57/MTA	Mouse antibody	Santa Cruz (sc-135746)	1:200 1:1000	IF WB
Prox1	Rabbit antibody	Abcam (ab199359)	1:200 1:1000	IF WB

IF = immunofluorescence, IP = immunoprecipitation, WB = Western blotting

4.3 Plasmids

In this study, Self-Inactivating (SIN) vector was used to transfer the genetic material to the target cells. Packaging plasmids pLp1, pLp2 and pLp/VSV-G were used together with pSIN plasmid carrying the gene of interest (Prox1 WT or MUT). Heterologous coat G glycoprotein from vesicular stomatitis virus (VSV-G) was used in place of the native HIV-1 envelope, and viral enhancer and promoter sequences have been deleted from SIN vectors. These modifications increase the safety of the lentiviral vector system.

N-terminal MYC-tagged Prox1 wild-type (WT) and DNA-binding deficient Prox1 mutant (MUT) were cloned into the pSIN lentiviral vector. Mutations N626A and N628A in Prox1 MUT were introduced by site-directed mutagenesis as described in (Petrova et al. 2002). Confirmation of mutations by sequencing (GATC Biotech, MyGATC) of the insert was performed to both Prox1 WT and MUT.

4.4 Virus production, infections, transduction and transfection

Wild-type wtKSHV was produced from TREx-RTA BCBL-1 cells stimulated with 1µg/mL doxycycline. After three days, the supernatant was filtered and the virus collected by centrifugation (SW32, 22,000 rpm at 4℃ for 2 h), and resuspended in PBS. Cells were infected with wtKSHV 20µg/mL in the presence of polybrene (Sigma H9268) 8µg/mL and spinoculated 450g for 30min at RT (Eppendorf Centrifuge 5804R). Polybrene acts by neutralizing the charge repulsion between cell surface sialic acid and virions, increasing infection efficiency (Davis et al. 2004).

Recombinant KSHV.219 was produced as described in (Vieira and O'Hearn 2004). Briefly, lytic reactivation was induced with 0.05µg/mL of doxycycline (Dox) (Sigma D9891) (Myoung and Ganem 2011). In addition, sodium butyrate (NaB) 3.3mM was used to enhance the reactivation (Vieira and O'Hearn 2004).

For production of lentiviral particles, HEK 293FT cells were transfected using Lipofectamine 2000 (Life Technologies) according to manufacturer's instructions with the packaging plasmids described above, and either with pSIN vector control (VC), Prox1

WT or MUT. Three days post-transfection virus was collected with PEG-it Virus precipitation (System Biosciences) according to manufacturer's instructions. iSLK.219 cells were transduced with lentiviruses in the presence of polybrene 8µg/mL. pSIN, Prox1 WT or MUT lentiviruses were used at a 1:500 dilution. Next, cells were spinoculated 450g for 30min at RT (Eppendorf Centrifuge 5804R) to increase the transduction efficiency.

4.5 RT-qPCR

Real time quantitative Polymerase chain reaction (RT-qPCR) was used to measure gene expression levels from extracted total RNA of the samples. Cells were collected from culture and extraction of RNA was performed using NucleoSpin RNA II kit (Macherey Nagel). The RNA concentration was measured with NanoDrop (Thermofisher). Using thermal MyCycler (Bio-Rad), total RNA was reverse-transcribed to cDNA with final reaction volume of 50 µl. cDNA synthesis was achieved using 1 µg of total RNA, 2.5µl dNTP (AB 1831597), 11.0µl MgCl2 (Thermo Scientific 00292623), 5.0µl 10X RT buffer (AB 1402114), 1.0µl RNAse inhibitor (AB 566718), 10.0µl Oligo (Invitrogen 1832147), and 1.25µl MultiScribe RT (Applied Biosystems 00472355) diluted in nuclease free H_2O up to volume of 19.3µl.

Transcription levels were measured in three technical replicates using Taqman Gene Expression Assays (Applied Biosystems) with unlabelled primers (listed in Table 2) and SYBR Green (2x) reaction mix (Fermentas) in the StepOnePlus Real Time PCR system (Applied Biosystems). The amplification was performed in LightCycler480 PCR 384 multiwell plate (Roche) with total volume of 10 µl per well, of which 2 µl cDNA and 8 µl mastermix. The PCR thermocycling included an initial denaturation at 95 °C for 10 minutes followed by 50 annealing cycles, each consisting of 15 seconds at 95 °C, 30 seconds at 60 °C and 30 seconds at 72 °C, and a final extension step at 72 °C for 7 minutes. Primer sequences used to amplify the indicated targets are listed in Table 2.

Table 2. Primers used in this study.

Target	Forward	Reverse
Actin	*TCACCCACACTGTGCCATCTACGA*	*CAGCGGAACCGCTCATTGCCAATGG*
K8.1	*AAAGCGTCCAGGCCACCACAGA*	*GGCAGAAAATGGCACACGGTTAC*
ORF45	*CCTCGTCGTCTGAAGGTGA*	*GGGATGGGTTAGTCAGGATG*
ORF57/MTA	*TGGACATTATGAAGGGCATCCTA*	*CGGGTTCGGACAATTGCT*
Prox1	*TGTTCACCAGCACACCCGCC*	*TCCTTCCTGCATTGCACTTCCCG*

4.6 Immunoblotting

The expression of selected proteins present in the cellular lysates were detected with Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting (WB).

Cells were lysed with RIPA buffer (150mM-NaCl; 1%-Igepal CA630-0.5% Na-deoxycholate-0.1% SDS-50mM; Tris-HCl-PH 7.8) supplemented with proteinase and phosphatase inhibitors (Pierce™ 88666, 88667), and cleared by centrifugation. The concentration of protein from each cleared sample was measured with Protein assay dye reagent concentrate (Bio-Rad) and BioPhotometer (Eppendorf), and accordingly used to obtain equal amount of protein in all the samples included in the experiment. Loading buffer 5XLaemmli was mixed with 2-mercaptoethanol and added to the cell extracts, which were boiled 5 min in 99°C. After cooling down at RT, samples and PageRuler Plus Prestained protein ladder (Thermo Scientific) were loaded on Criterion TGX precast polyacrylamide gel (Bio-Rad), and run at 55mA for approximately 40 min. After the electrophoresis run, proteins in the gel were transferred to nitrocellulose membrane (Bio-Rad) using trans-blot Turbo Transfer system (Bio-Rad).

Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline with 0,1% Tween (TBS-T) solution for 1h in rocking table. Primary antibody incubations were performed in the blocking solution overnight (O/N) at 4°C. After, the membranes were washed and appropriate HRP-conjugated lgG secondary antibodies (Cell Signalling, Millipore) were incubated for 1h in blocking solution at RT. The membranes were washed again and the rocking table was used in all the incubations and washes. Antibodies used

in this study are listed in Table 1. The luminescent signal from immunoblotted proteins was detected using WesternBright Sirius detection kit (Advansta) and ChemiDoc MP imaging system (Bio-Rad). The relative expression of proteins was determined from generated images, and β-actin (42 kDa) was used as a loading control.

4.7 Immunofluorescence staining

Immunofluorescence (IF) staining was performed in 96-well black bottom plate (Perkin Elmer). After fixing cells with 4% PFA for 20min in RT, cells were washed 3x with 0,5% bovine serum albumin (BSA) in PBS to block unspecific staining. Permeabilization was done with 0,2% Triton-X 100 (Sigma SLBD7186V) simultaneously with nuclei counterstaining using 1ug/mL of Hoechst 33342 (Fluka Biochemika 14533) in PBS for 10 min dark incubation at RT. Between all incubations, the cells were washed 3x with 0,5% BSA in PBS. Primary antibodies for specific staining of proteins were incubated in the dark for 1h at RT. Appropriate secondary antibodies conjugated to Alexa Fluor 488, 594, 647 stains obtained from Life Technologies (Thermofisher) were incubated with 0,5% BSA in PBS in dilution of 1:1000 for 1h at RT. Finally, the cells were washed 2x with 0,5% BSA in PBS and once with only PBS before imaging. Thermo Scientific Cell Insight Hight Content Screening system provided by Biomedicum Imaging Unit (BIU) was used to image immunofluorescence signal from the plates. Antibodies used in the study are presented in Table 1.

4.8 Co-immunoprecipitation

For immunoprecipitations, cells were lysed in the IP lysis buffer (150mM-NaCl; 50mM-Tris-HCl-pH 7.8; 0.2%-NP40 Igepal; 1% glycerol; 0.5mM-EDTA) supplemented with complete proteinase and phosphatase inhibitory cocktails, and the whole cell extracts were cleared by centrifugation for 20min at 4℃. Next, aliquots were taken for inputs and the rest of the extracts were incubated either with a MYC antibody (to immunoprecipitate Prox1), or a control mouse lgG antibody O/N in 4℃ rotating. Protein G beads (Abcam) were washed with IP buffer and added (50µl) into the samples for 2h 4℃, followed by a

wash with IP buffer. Immunoprecipitated and total proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes for WB. Antibodies used in the study are listed in the Table 1.

4.9 Statistical analysis

For statistical evaluation of qRT-PCR data, the logarithmic values were converted to linear log2 scale values by using double delta C_T (2-ΔΔ C_T) method, designed to analyse the relative changes in gene expression. Threshold cycle (C_T) is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold (Livak and Schmittgen 2001). Reference gene *β-actin*, used as internal control, was accounted in the calculations to correct differences in quality, in cDNA and PCR synthesis efficacy between samples. Graphical representations and statistical analysis were generated with GraphPad Prism Software v6.0c with RT q-PCR data presented as the means ± standard deviation (SD). Differences between means were analysed with One-way ANOVA test corrected for multiple comparisons, Prox1 pSIN WT, MUT and mock were compared to pSIN VC. A difference between two means was considered significant when *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

For statistical evaluation of virus release assay, the virus loads were measured as percentage of GFP -positive (virus-infected) cells relative to the number of nuclei. Quantification was carried out with CellProfiler Software v2.1.1 and output of 2 biological replicates was processed by calculating average of infected cells of every dilution from 3 technical replicates all having 9 images. Dilution having infected cells between 10-20% of the population was selected for statistical analysis as with this percentage of GFP-positive (virus-infected) cells it is possible to better appreciate and quantify the differences among the different samples. Graphical representations and statistical analysis were generated with GraphPad Prism Software v6.0c and data presented as the means ± standard deviation (SD). Differences between means were indicated as p-values tested with One-way ANOVA for multiple comparisons, all conditions were compared to pSIN and mock. A difference between two means was considered significant when **p < 0.01, ****p < 0.0001.

5 Results

5.1 Prox1 increases lytic gene expression

To elucidate the role of Prox1 during KSHV reactivation, the levels of lytic transcripts both during latency and during lytic reactivation in cells ectopically expressing of Prox1, was measured. The study was conducted in iSLK.219 cells, which do not endogenously express Prox1 and are stably infected with the recombinant virus. To this end, iSLK.219 cells were seeded on a 24-well plate and the next day transduced as described with lentivirus coding for Prox1 WT or MUT, unable to bind DNA. In addition, pSIN VC and polybrene-treated cells (mock) were used as a negative control. A day after, cells were left untreated (latent) or reactivated with Dox. Cells induced were incubated for 48h, and RNA extraction and gene expression measurement by RT-qPCR were performed. Prox1 expression is relatively high in Prox1 WT and MUT, but not in the mock, indicating successful transduction (Figure 9). Prox1 MUT expression is higher than Prox1 WT even though the transduction was performed in the same way and with the same pSIN vector concentration.

Data obtained from RT-qPCR indicate that an increase in lytic gene expression is already detectable during latency in the presence of Prox1 WT (Figure 9a). This is surprising since no RFP, which the recombinant virus expresses upon reactivation, was detected in Prox1 WT expressing iSLK.219 prior to Dox treatment. In contrast, Prox1 MUT-expressing cells had very low levels of lytic mRNAs in the absence of Dox, probably due to the dominant negative effect. After the induction of lytic reactivation by Dox, enrichment in the relative mRNA levels of lytic markers was observed, differing significantly between Prox1 WT and the controls, (Figure 9b). Relative expression levels of viral immediate-early ORF45 and early ORF57 are higher in the presence of Prox1 WT compared to other conditions. Especially the mRNA level of late expressed K8.1 is significantly increased with Prox1 WT, up to 13-fold compared to mock (Figure 9b). The observation that similar mRNA levels of lytic markers and Prox1 between pSIN VC and the mock ensures that pSIN does not have significant effect on relative expression of lytic markers or on the endogenous level of Prox1. It is important to take into consideration

that the relative latent and lytic relative expression levels are not comparable (Figure 9a and b). Thus, latent controls (pSIN and mock) express very low levels of lytic markers, allowing the detection of even a slight increase in the lytic gene expression of Prox1 WT. On the other hand, the lytic controls, VC and mock, themselves yield moderate levels of lytic gene expression after reactivation, meaning that lytic gene expression levels of Prox1 WT are indeed very high.

In conclusion, the gene expression analysis suggests Prox1 as a positive regulator of KSHV lytic gene expression. The increased lytic gene expression in both latent and reactivated cells indicate Prox1 having a role in both phases of the KSHV life cycle. However, the effect of Prox1 on viral lytic expression prior to and after the reactivation needed to be studied also at a protein level.

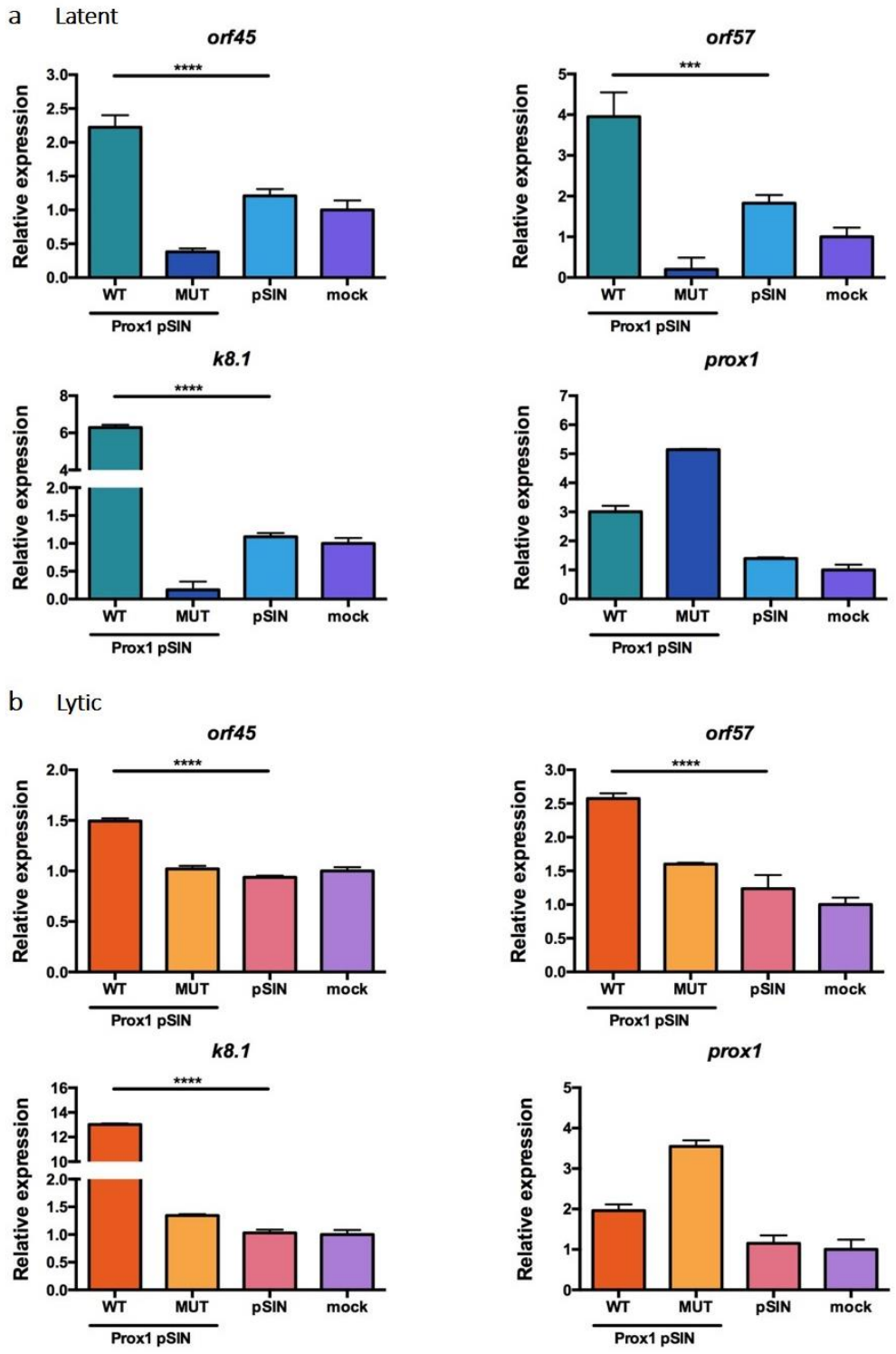

Figure 9. Prox1 ectopic expression increases lytic gene expression a, b. iSLK.219 seeded at a density of $1.25x10^5$ cells per well in a 24-well plate and transduced with pSIN Prox1 WT, MUT or pSIN VC or treated with polybrene only (mock), **a**. untreated (latent) and **b**. reactivated with Dox 24h (lytic), mRNA levels of indicated transcripts normalized to *β-actin* are shown. Bars indicate mean ± SD across 3 different replicates. ns = non-significant, ***p < 0.001, ****p < 0.0001.

5.2 Prox1 increases lytic protein expression

To further study the effect of Prox1on viral lytic protein expression, immunoblotting was performed to study expression of selected lytic markers at protein level in latent and reactivated iSLK.219 cells. Cells were seeded and transduced the next day either with Prox1 WT or MUT as described above. Reactivation was performed the following day either with only Dox or using Dox in combination with NaB to achieve maximal level of reactivation. Polybrene-treated cells (mock) was used as a negative control. Cells induced with Dox+NaB were incubated for 24h, and cells induced only with Dox for 48h. The cells were harvested in RIPA buffer and processed for SDS-PAGE and Western blotting. Viral protein expressions of selected latent and lytic markers in addition to Prox1, to assess the efficiency of transduction, and β-actin as a loading control were analysed.

The results from RT-qPCR (Figure 9) showed differences in the expression of lytic markers prior to reactivation, however, this was not observed in the immunoblot (Figure 10). The difference is likely due to RT-qPCR being more sensitive in detecting low levels of gene expression compared to immunoblotting. After reactivation, the specific bands for the selected lytic proteins ORF45 (60-80kDa), ORF57 (55kDa) and K8.1 (40kDa) are visible. Expression of both, ORF45 and ORF57, did not indicate a clear difference between Prox1 WT and MUT. However, the expression of the structural envelope protein K8.1 was substantially more abundant upon Prox1 WT expression. Interestingly, even though Prox1 WT expressing cells have the highest lytic protein expression, it was also relatively high with Prox1 MUT but not in the mock. This indicates that even though Prox1 MUT is devoid of DNA binding activity, it can promote lytic protein expression. However, the lytic promoting effect can be detected only at a protein level, as increase in lytic markers was not observed at transcript levels (Figure 9b).

Latency-associated nuclear antigen LANA is encoded by ORF73 throughout the KSHV life cycle, in both latent and lytic phases. This was confirmed in the LANA blot showing a series of bands between 230 and 200 kDa (Figure 10a). As the name implies, the main function of LANA is the viral episome maintenance, and it is localized in the nucleus of latently infected cells (Ballestas, Chatis, and Kaye 1999). However, the third and the lowest bands detected in the reactivated cells represent most likely the recently

described cytosolic LANA (Toptan et al. 2013; Zhang et al. 2016). Observed only during lytic cycle, these N-terminally truncated isoforms of LANA are present in the perinuclear and cytoplasmic sites of KSHV-infected cells. Again, Prox1 MUT expression is higher than Prox1 WT as it is more stable than WT, due to slower degradation and resulting in higher protein levels (Dr. Silvia Gramolelli, personal communication). Expression of Prox1 (80-100kDa) is strongly downregulated after the reactivation (Figure 10a).

iSLK.219 can be induced to reactivation with Dox only (Figure 10a), however, more efficient induction of reactivation by addition of NaB shows a strong enrichment of lytic proteins, also in the mock (Figure 10b). Enhancement of reactivation with NaB causes such a strong viral protein expression that differences in the levels of ORF45 and ORF57 are not anymore detectable. Nevertheless, the protein expression of K8.1, translated later in the lytic replication cycle, is distinctly higher with Prox1 WT compared to Prox1 MUT and mock. Taken together, these results are consistent with the lytic mRNA expression analysis and implicate that Prox1 has a promoting effect on KSHV lytic protein expression.

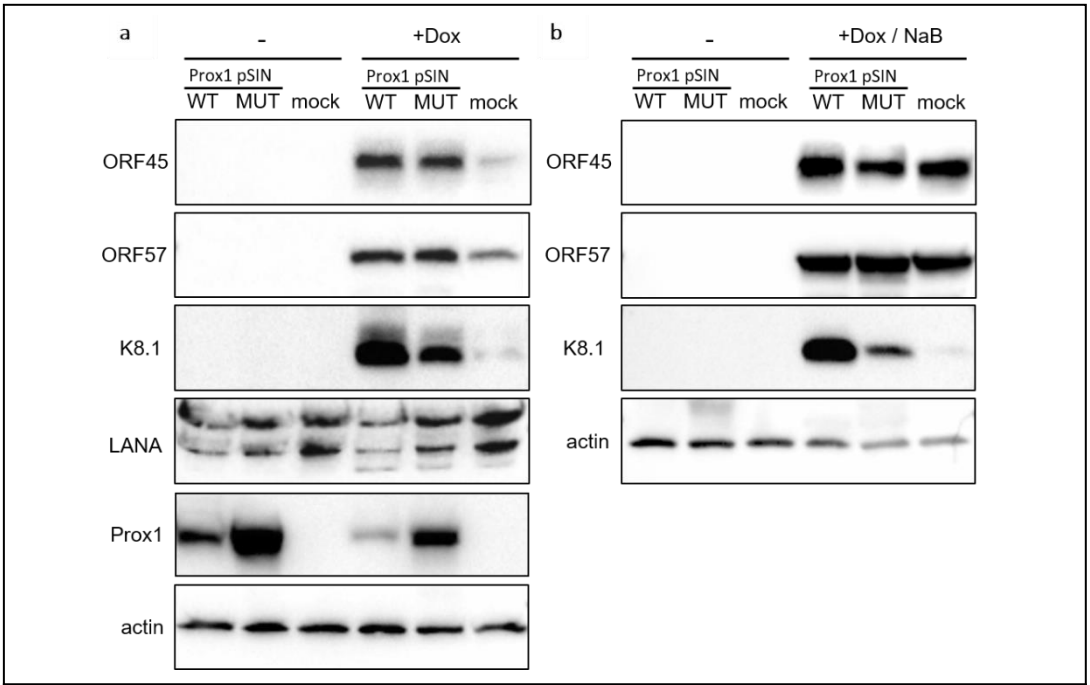

Figure 10. Prox1 ectopic expression increases lytic protein expression a, b.
iSLK.219 seeded at a density of $5x10^5$ cells per well in a 6-well plate (Greiner bio-one) and transduced with Prox1 pSIN WT or MUT or left untransduced (mock), left blots: untreated (latent; indicated with -) and right: reactivated with Dox for 48h (**a**) or Dox/Nab 24h (**b**), protein expression of Prox1 and viral markers with β-actin as loading control were analysed by immunoblot.

5.3 Prox1 enhances lytic reactivation

The effect of Prox1 on lytic reactivation was studied in more detail using iSLK cells infected with the recombinant virus rKSHV.219 which in latency expresses GFP and upon reactivation also expresses RFP (Vieira and O'Hearn 2004). iSLK.219 rKSHV cells were seeded on black bottom 96-well plate and the next day, cells were transduced with pSIN lentivirus either empty or expressing Prox1 WT or a mutant, which does not bind to DNA. The virus was reactivated by inducing RTA expression upon Dox treatment for 24h. Reactivation levels (RFP) of iSLK.219 cells, ectopically expressing either Prox1 WT or MUT, were compared to pSIN VC. In addition, selected lytic markers and latency-associated LANA were detected with immunofluorescence staining to observe the reactivation at single cell level.

By observing differences in the number of RFP positive cells by high-throughput images, shown in Figure 11, cells expressing Prox1 WT display the highest reactivation levels. Immunofluorescence signal of viral nuclear protein ORF57 can be observed in slightly higher abundancy upon Prox1 WT ectopic expression (Figure 11). More striking differentially expressed lytic protein in Prox1 WT-expressing iSLK.219 cells is K8.1, structural envelope glycoprotein incorporated to virions (Birkmann et al. 2001). Viral latent gene product LANA is present in the nuclei of infected cells and expressed in all conditions (Ballestas, Chatis, and Kaye 1999). The efficacy of Prox1 introduction by transduction can be observed from staining with Prox1-specific antibody. As seen from Figure 11, Prox1 is present only in WT and MUT, but not in pSIN VC or mock and Prox1 MUT expression is higher than Prox1 WT.

This lytic reactivation assay further indicates Prox1 as a positive regulator of KSHV reactivation from latency. Reactivation through activation of RTA initiates the lytic replication cycle and the expression of lytic markers downstream of RTA is stronger in the presence of Prox1.

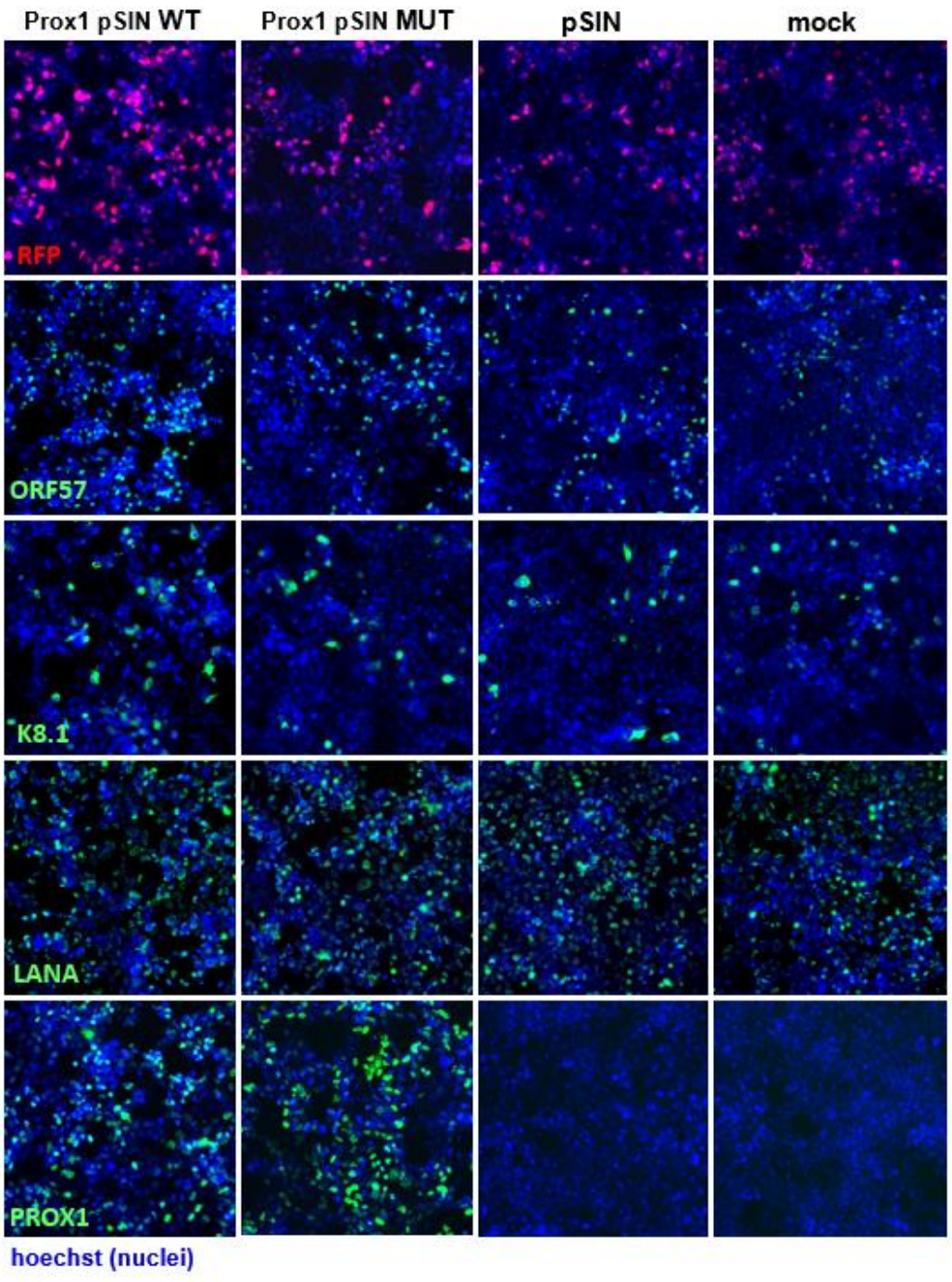

Figure 11. Prox1 ectopic expression increases rate of lytic reactivation and expression of lytic markers. iSLK.219 seeded on black bottom 96-well plate (Perkin Elmer) at density of $5x10^3$ cells per well were transduced with pSIN prox1 WT, MUT or pSIN, and reactivated with Dox for 24h were fixed and stained with the indicated antibodies (green) nuclei were counterstained with Hoechst 33342 (blue). Lytic cells are visualized as RFP positive cells (red). Representative images

5.4 Prox1 enhances KSHV release of infectious virions

Previous results have revealed that Prox1 is a positive regulator of lytic reactivation enhancing both the lytic gene and protein expression. The successful completion of the lytic cascade leads to the assembly and release of newly synthesized viral particles able to infect new target cells. Therefore, we next asked whether ectopic expression of Prox1 WT or MUT would lead to higher viral yields, quantitatively measured in terms of efficacy in infecting naïve cells.

A day before seeded iSLK.219 cells were either left untransduced (mock) or transduced with pSIN Prox1 WT or MUT or pSIN VC and 24h later reactivated as indicated. Reactivation was verified by measuring the RFP signal under a fluorescent microscope (data not shown). Cells induced with the combination of Dox and NaB were incubated for 48h, and cells induced only with Dox for 72h. The virus-containing supernatant was then collected and used to infect naïve U2OS cells seeded the day before on a 96-well plate. Infection by supernatant was done as serial dilution in the presence of polybrene to enhance the infectivity. Schematic representation of the virus release assay workflow is depicted in Figure 12. After incubating 24h, GFP intensity was analysed in the target U2OS cells. The signal of GFP was enhanced with immunofluorescence staining and nuclei were visualized with Hoechst 33342 (Figure 13a, c). Virus loads were measured as a percentage of GFP -positive target cells from all the detected cells (Figure 13b, d).

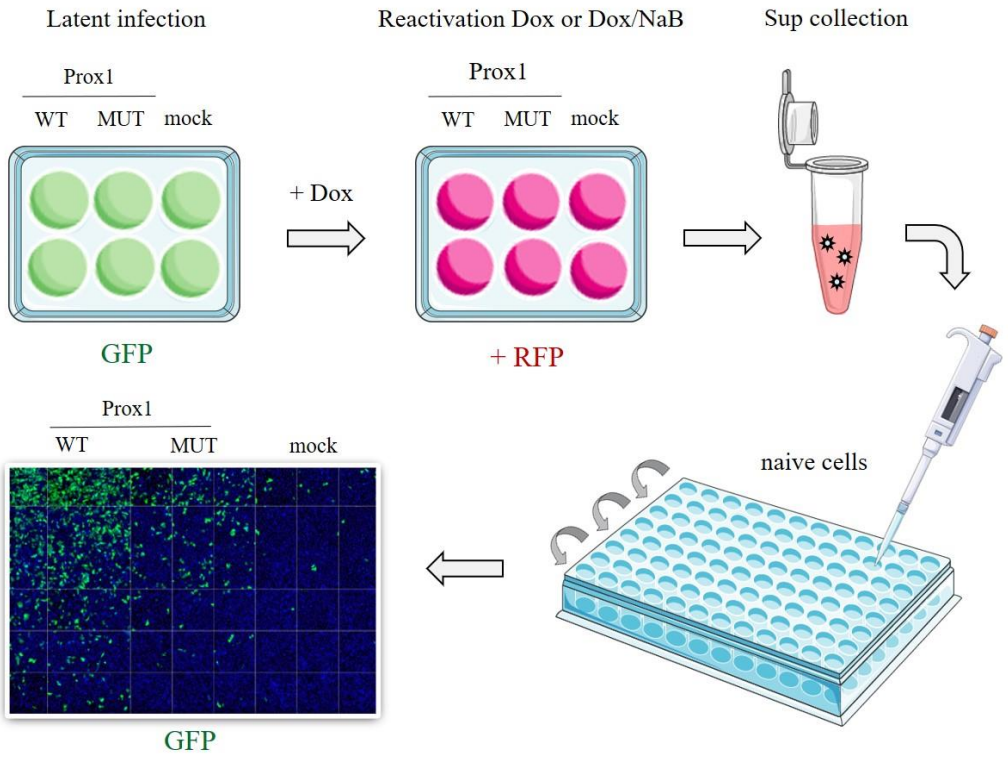

Figure 12. Schematic representation of virus release assay workflow. Latent iSLK rKSHV seeded at a density of $5x10^5$ cells per well in a 6-well plate (Greiner bio-one) were transduced with Prox1 pSIN WT or MUT, and mock, depicted with GFP (green) and treated with Dox or Dox+NaB for reactivation depicted as RFP (red). Viral particles were shed to the culture media, and the supernatant was used to latently infect U2OS, seeded day before at a density of $8x10^3$ cells per well in a 96-well plate (Perkin Elmer), in serial dilution. Virus loads are measured as GFP% (infected)-positive cells from data obtained from high-throughput immunofluorescent images.

Quantification of the GFP-positive cells from the whole cell population calculated as a percentage of infected cells revealed that Prox1 WT significantly increased the virus release, more than 10-fold (Figure 13b, d). Addition of NaB enhances the reactivation causing increase of newly infected cells from 14% to 90% of cells when compared to cells infected with a sup from reactivation done only with Dox. Due to a very high infection rate, it is more reliable to quantify the virus release from cells infected with the more diluted supernatant (Figure 13c area indicated in red). Furthermore, enhancement with NaB causes quite a strong increase in the virus release of reactivated cells with Prox1 MUT (Figure 13d), not observed in cells reactivated using only Dox (Figure 13b), that represents a more reliable way to observe and interpret the virus release results.

In conclusion, Prox1 WT, but not MUT, significantly increased the release of infectious virions and consequently the infection rate of naïve cells. Hereby the virus release assay indicates that infected cells expressing Prox1 produce more viral progeny, resulting in more efficient dissemination and infection of new host cells.

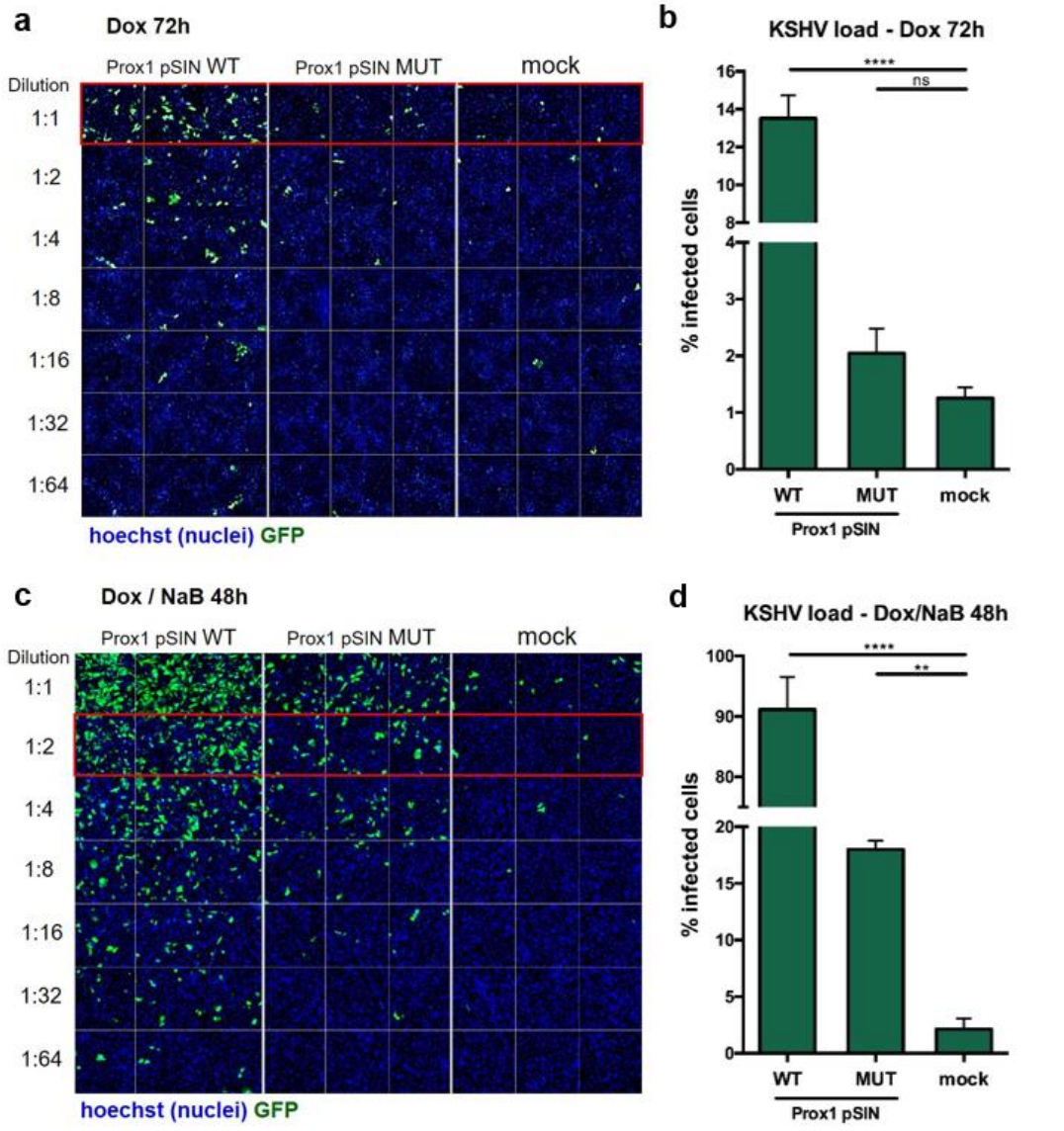

Figure 13. Prox1 ectopic expression enhances KSHV release a-d. KSHV loads of U2OS infected with serial dilutions of supernatant from iSLK rKSHV transduced as indicated and treated with **a, b** Dox 72h or **c, d** Dox+NaB 48h is shown. **a, c.** High-throughput images of infected U2OS cells, with each square representing one well. Red areas highlight the dilution used in analysis. **b, d.** Bars indicate average virus loads measured as GFP% (infected)-positive cells shown as mean ± SD 9 images/condition across 3 different replicates. ns = non-significant, **p < 0.01, ****p < 0.0001.

5.5 Time course of Prox1 expression during KSHV primary infection

To investigate the expression levels of Prox1 during the KSHV infection cycle, infection kinetics assay was performed. U2OS cells infected with wtKSHV and followed during the indicated time-scale from the onset of KSHV infection to latent infection establishment. Prior to infection, lysate of uninfected cells (timepoint 0h) was harvested in RIPA buffer. Infected cells were harvested at 8 ,12, and 24h and after 2, 3, 4 and 6 days (d). The expression of Prox1, KSHV IE gene RTA and LANA were measured by immunoblotting with β-actin as a loading control. The results are shown in Figure 14.

U2OS cells endogenously express Prox1 at very low, nearly undetectable levels prior to infection (Dr. Gramolelli, personal communication, Human protein atlas). Viral proteins are not expressed at 0h as the sample was obtained before addition of infectious particles. After the infection with wtKSHV, expression of Prox1 increases rapidly within a day reaching a peak concurrently with RTA (110-130 kDa) at 24h post infection (p.i.) (Figure 14). The expression of LANA progressively but moderately increases and likely represses the RTA promoter to establish latency and to maintain episomes in the newly infected cells, as described before (Chen et al. 2001; Lan et al. 2005). Most importantly, after the lytic burst, Prox1 expression progressively decreases. Similarly, the downregulation of Prox1 48h after reactivation was observed in a previous assay (Figure 10a). Within a week, as the acute infection has passed and latency is established by increased expression of LANA, Prox1 is downregulated simultaneously.

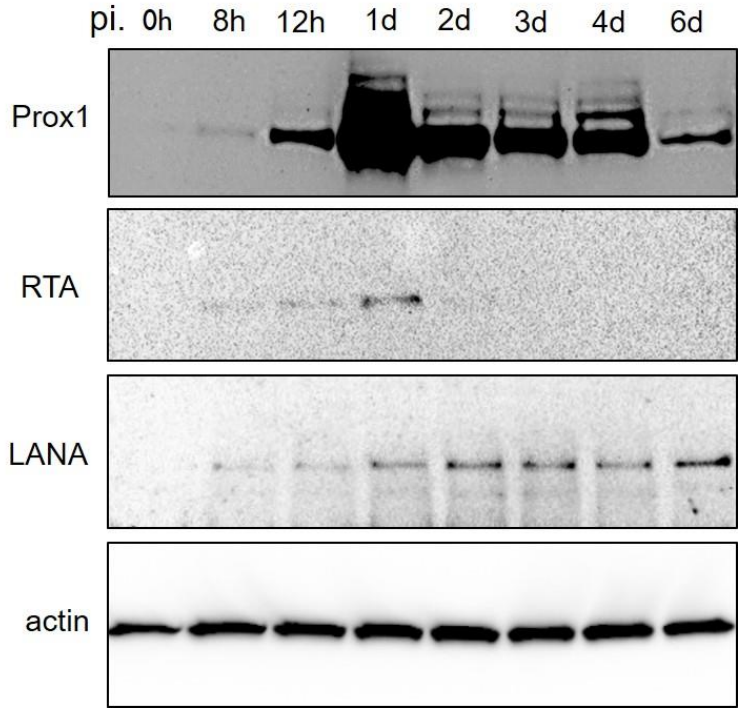

Figure 14. Prox1 expression kinetics during KSHV primary infection. U2OS cells, seeded at a density of $1.25x10^5$ cells per well in a 24-well plate were infected with wtKSHV and harvested at the indicated time post-infection (p.i.). Whole cell lysates were analysed by immunoblot for the expression of the indicated proteins.

5.6 Co-immunoprecipitation of Prox1 and RTA

Since our data indicated that Prox1 enhances the RTA-dependent reactivation and transcription of KSHV lytic genes, we asked whether Prox1 achieves this by interacting with RTA. To this end, co-immunoprecipitation of Prox1 and RTA was performed. iSLK.219 cells, seeded on a 6-well plate, were transduced with pSIN lentivirus expressing Prox1 WT or MUT and reactivated by inducing RTA expression with Dox treatment for 24h, as described above. The cells were harvested in IP buffer and processed for co-immunoprecipitation and immunoblotting.

The obtained data indicates that RTA was immunoprecipitated with Prox1 WT, but only weakly with Prox1 MUT, devoid of the DNA binding activity (Figure 15), suggesting that Prox1 and RTA interact with each other. However, these results are

preliminary data and more careful investigation will be conducted to further asses the potential binding of Prox1 and RTA.

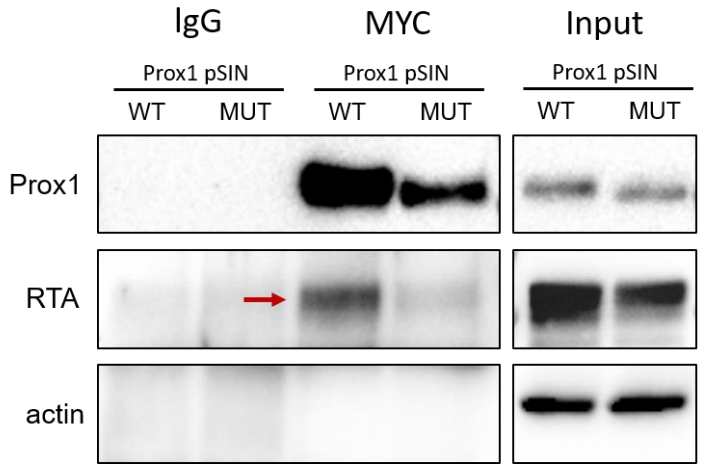

Figure 15. RTA is co-immunoprecipitated with Prox1.
iSLK.219 cells transduced with Prox1 pSIN WT or MUT and reactivated with Dox for 24h were subjected to immunoprecipitation by anti-MYC or control IgG and analysed by immunoblotting with the indicated antibodies. Actin was used as a loading control. Red arrow highlights the RTA immunoprecipitated by Prox1 WT.

6 Discussion

Oncogenic viruses, such as KSHV, provide unique experimental models to investigate the molecular biology of tumorigenesis. Oncovirology has been of immense importance in the discovery of tumor suppressor genes and oncogenes, and to our understanding of their critical role in cancer development and progression (Vogt 2012; Chang, Moore, and Weiss 2017). The careful study of KSHV oncogenes elucidates their role in the lifecycle of KSHV and capacity to induce cell transformation via interaction with the host-signaling pathways, such as Prox1.

In vitro, the expression of Prox1 is altered upon KSHV infection of ECs (Hong et al. 2004; Wang, Trotter, et al. 2004). KSHV-mediated regulation of Prox1 is a crucial step in the EC fate reprogramming, enabling endothelial lineages to acquire mixed identity resembling that of SCs of advanced KS lesions (Yoo et al. 2012). Both viral genes and host signaling pathways have been shown to take part in the regulation of Prox1 in the infected ECs (Emuss et al. 2009; Yoo et al. 2010; Cheng et al. 2011; Yoo et al. 2012; Chang and Ganem 2013). However, more detailed host-virus interactions contributing to the Prox1 expression changes need to be investigated, in addition to the underlying motive of this manipulation.

Viral transcriptome analysis has revealed KSHV-infected LEC to displays dysregulated expression of numerous lytic cycle genes, indicating spontaneous reactivation in the context of continued cell survival and proliferation (Chang and Ganem 2013). This study explores the contribution of Prox1 ectopic expression to KSHV lytic reactivation and stems out from our preliminary data on KSHV-infected LECs depleted of Prox1 which displayed a reduced spontaneous lytic reactivation. Until now, the role of Prox1 ectopic expression in reactivation of KSHV had not been defined. We are the first ones to show that Prox1 positively regulates KSHV reactivation by enhancing lytic gene and protein expression as well as increasing the release of infectious progeny (Figure 16). The promoting role of Prox1 in the RTA-dependent reactivation and transcription of KSHV lytic genes is potentially mediated by the interaction of Prox1 and RTA.

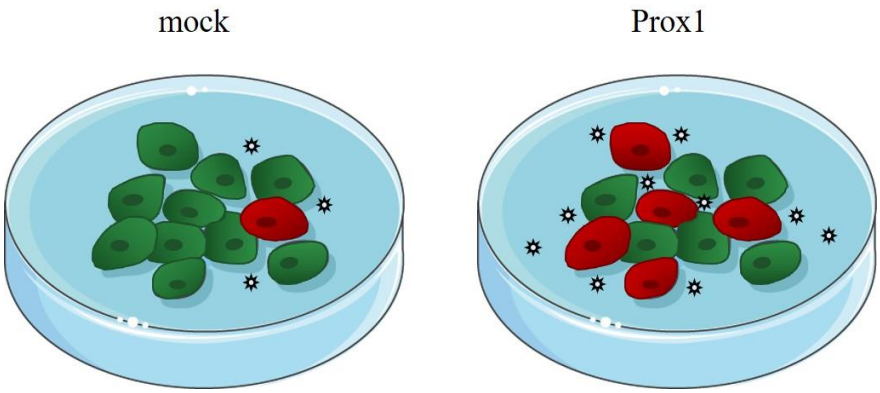

Figure 16. Prox1 is a positive regulator of KSHV lytic cycle. iSLK cells infected with rKSHV.219 express GFP in latency and RFP upon reactivation (Vieira and O'Hearn 2004). In this study, upon reactivation with Dox, iSLK.219 cells transduced with Prox1 demonstrate increased reactivation rate, lytic gene and protein expression and also increased virion production compared to the negative control (mock).

6.1 KSHV and Prox1 manipulation – beyond EC reprogramming

Endothelial reprogramming is a key factor in KS pathogenesis resulting in characteristic spindle cell. Yet it has been pondered why KSHV induces cell fate reprogramming of its host, whether it is a mere by-product or the goal of the viral infection, providing pathological advantage to KS tumorigenesis, is not known. Considering that viruses are dependent on their hosts to survive and reproduce, most likely it is not the goal of the virus to kill its host by KS manifestation. Loss of balance between the virus and the host has been shown to occur upon immunodeficiency in addition to virus-host interactions, promoting pathological oncogenesis.

Basal, low expression of Prox1 in mature BECs is upregulated after infection, and the normally high level of Prox1 expression in mature LECs is downregulated after infection (Hong et al. 2004; Wang, Trotter, et al. 2004). It has been shown that BECs are not competent host for the virus. In the light of our results, one could hypothesize that Prox1 is needed to enhance the reactivation and to maintain the population of latently infected cells. In infected LECs, however, Prox1 is downregulated after the acute infection possibly to restrict excessive reactivation and to facilitate the establishment of latency. As the results of this thesis demonstrate Prox1 to enhance KSHV reactivation in other cells than ECs, the virus might manipulate Prox1 expression in order to ensure adequate

levels of lytic reactivation rather than induce a specific differentiation state in the cells. In the midst of finding the right balance of Prox1 for viral replication, the expression changes in infected ECs could cause ECs to be reprogrammed towards the pathogenic SC type with tumorigenic abilities. Thus, the reprogramming of LECs resulting from the manipulation of Prox1 by KSHV may be just a side effect.

On the other hand, KSHV induces reprogramming of LECs towards a mesenchymal phenotype via endothelial-to-mesenchymal transition (EndMT), endowing LECs with invasive properties by inducing MMP-14 upregulation (Cheng et al. 2011). vFLIP and vGPCR were shown to initiate the EndMT of KSHV-infected LECs through activation of the Notch pathway. EndMT enables cells to detach from the organised EC layer and acquire invasive and migratory properties (Potenta, Zeisberg, and Kalluri 2008), whereas MMP-14 is a key regulator of tumor cell growth and dissemination (Rowe and Weiss 2009). The gain of MMP-14 dependent invasive properties enhance the oncogenic potential of infected LECs, referring to deliberative reprogramming towards thriving, however pathogenic cell type. Moreover, our recent studies have revealed Prox1 to negatively regulate MMP14 levels by binding to the MMP-14 promoter and repressing its transcription (Gramolelli S. manuscript in revision). Depletion of Prox1 in vivo increased MMP-14 expression in murine and in human LECs, promoting invasiveness seen as increased endothelial sprouting in a 3D invasion assay.

Taken together, after the primary KSHV infection or reactivation from latency in already infected cells, Prox1 might be first upregulated to act as positive regulator of lytic replication, and later downregulated to facilitate the establishment of latency, and in the process the virus potentially induces LEC reprogramming and acquisition of oncogenic properties.

6.2 Prox1 and KSHV - a possible dual effect on reactivation

Being a transcription factor, Prox1 regulates the expression of other genes via binding to DNA. Prox1 can act both as a repressor and an enhancer of gene expression (Elsir et al. 2012) and has an important role in mediating the effects of KSHV on EC

reprogramming. Upon KSHV infection Prox1 expression is upregulated in BECs and downregulated in LECs, sufficiently reprogramming distinct endothelial lineages to acquire mixed phenotype (Yoo et al. 2012).

The results of this thesis show that Prox1 WT but not its mutant, devoid of DNA binding activity, could increase viral gene expression (Figure 9). These findings indicate that lytic promoting effect on gene transcription depends on the DNA binding ability of Prox1. Potentially Prox1 might bind to the promoter of lytic genes and enhance their expression, a conjecture remaining to be demonstrated in the future studies. However, at a protein level, Prox1 MUT has a positive effect on expression of lytic markers (Figure 10) and in addition, Prox1 MUT moderately promotes virus production and release (Figure 13). A possible explanation for this is that Prox1 MUT can enhance lytic protein expression in a yet unknown DNA-binding-independent manner which remains to be investigated. Furthermore, in the experiments of this study Prox1 MUT expression was higher than Prox1 WT as Prox1 MUT is more stable resulting in a slower degradation compared to Prox1 WT. Might be, that besides being a transcription factor, Prox1 could regulate the degradation of proteins. This, however, remains to be investigated.

After the reactivation of KSHV through the induction of RTA expression, the lytic replication cycle is initiated and downstream targets of RTA are expressed in a precise temporal cascade. Prox1 is promoting the lytic protein expression only after reactivation (Figure 10), whereas the more sensitive RT-qPCR detects an increase in the expression of the selected lytic genes already in latent cells upon ectopic Prox1 WT expression (Figure 9a). This may result from the spontaneous reactivation of very few cells prior induction or Prox1 might promote infected cells to prepare in unknown manner for the coming reactivation already during latency. This, however, is debatable and should not be interpreted as such before more careful investigation. RTA has been shown to be necessary for reactivation and initiation of lytic replication, indicating that Prox1 would need to the presence of RTA to cause the weak expression of lytic genes.

Infection of U2OS cells with wtKSHV triggered a rapid increase of Prox1 protein expression peaking concurrently with RTA. Our ongoing studies have implicated Prox1 and RTA to co-localize into the nuclei of infected and reactivated cells, and further that

Prox1 binds to RTA and enhances RTA to transactivate its own promoter as well as other KSHV lytic promoters (Figure 15). After the early peak, Prox1 expression progressively decreased and LANA was upregulated (Figure 14). This may imply that LANA could have a repressing role on Prox1, by downregulating its promoter to facilitate latency establishment. In addition to the repressing role on RTA (Lan et al. 2005; Chen et al. 2001), LANA might downregulate Prox1 to suppress its promoting effect in reactivation. However, this remains to be demonstrated by further experimental studies.

Taken together, this thesis shows for the first time that Prox1 is differentially regulated by KSHV in cell types other than LECs and BECs. Indeed, KSHV engages in adjusting Prox1 expression level upon infection of ECs, further suggesting a crucial role of Prox1 in the KSHV life cycle.

6.3 Translational prospects

The research on KSHV pathogenesis and development of KS is complicated due to the lack of suitable animal models and an endothelial cell transformation system. Also, *in vitro* cell culture models with cells expressing viral genes in isolation tend to give more dramatic phenotypes than those of KSHV itself, inviting misinterpretation. Nevertheless, experimental data should be interpreted in the context of the pathobiology of KSHV and comparison to clinical behavior of KS should be taken into consideration.

However, an improved understanding of KSHV pathobiology endows possibilities for the development of potential targets for prevention and intervention of KSHV induced oncogenesis. Novel findings of KSHV reactivation could be exploited in targeting latent cells to reactivate, as latently-infected cells are insensitive to anti-herpesviral drugs targeting only viruses in the lytic phase, when the viral DNA is replicated (Kedes and Ganem 1997; Antman and Chang 2000). Elucidating the role of cellular factors manipulated by KSHV infection, such as Prox1, in viral reactivation and lytic replication could alleviate the health burden of KS and other lymphoproliferative diseases through translational therapeutic strategies.

7 Conclusions and future perspectives

The regulation of Prox1 is altered during the KSHV infection and changes in the Prox1 expression has an effect on the viral life cycle. The results of this thesis suggest that Prox1 is a positive regulator of KSHV lytic reactivation from latency. Ectopic expression of Prox1 enhances the lytic replication and expression of lytic genes needed for the production of viral particles to be released from the infected host cell. This prompts further interest to elucidate the underlying mechanism of Prox1 to promote KSHV reactivation. Modulation of reactivation by Prox1 possibly results from its interaction with RTA, the KSHV-encoded transcription factor necessary and sufficient for the initiation of lytic replication. In the future, we will continue investigating the function of Prox1 and RTA in the KSHV reactivation.

Prox1 has been shown to be altered after KSHV infection of LECs and BECs, however the kinetics of differential regulation of Prox1 expression in other cell lines has not been addressed. Results of this thesis suggest that KSHV modulates Prox1 expression levels in the infected cells according to its needs during the course of infection. In the future, the plan is to elucidate the expression modulation of Prox1 upon prolonged infection in more detail. We are determined to address whether the expression of Prox1 eventually returns to the basal low level seen prior to the infection and to test this in other cell lines, especially in LECs. This thesis shows that Prox1 expression is downregulated after its strong induction during the lytic burst, however, the mechanisms and factors behind this remain be investigated.

Development of KS following the KSHV infection is a complicated multistep process, where the virus-host interactions play a critical role in the signaling transduction, immune evasion and in the changes of the host cell identity to acquire tumorigenic abilities. Prox1, as one of these host factors, has an immense role in the life cycle of KSHV and in the virus-induced oncogenesis of KS.

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